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# Monocyte Ingestion of IgG-Coated Erythrocytes from Haemolysing and Non-Haemolysing Subjects

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*Key Words* Antibodies Autoimmune haemolysis Erythrocyte phagocytosis Haemolytic anaemia Monocytes

*Abstract* A number of individuals in the population, including healthy blood donors and patients on methyldopa, show evidence of erythrocyte coating by IgG autoantibodies (positive direct Coombs test) without signs of a haemolytic state The readiness with which red cells from these subjects are taken up by normal human monocytes in short term culture was examined and compared with the phagocytosis of red cells from cases of IgG mediated haemolytic anaemia Red cells from non haemolysing subjects were as avidly ingested in this system as were red cells from haemolysing patients Similar results were obtained using normal red cells sensitised with autoantibody eluted from the cells of a healthy blood donor

The majority of IgG erythrocyte antibodies are unable to initiate complement activation They can only cause haemolysis via the agency of mononuclear phagocytic cells These cells, which are localised predominantly in the liver and spleen, carry membrane receptors for the Fc region of IgG enabling them to bind and subsequently engulf antibody-coated red cells [1, 2] Apparently identical Fc receptors are carried on circulating blood monocytes, the immediate precursors of tissue phagocytes The demonstration of this functional similarity between monocytes and tissue histiocytes has led to proposals that the *in vitro* interaction between isolated blood monocytes and antibody-sensitised red cells provides a valuable model for the mechanism of *in vivo* haemolysis [1, 3, 4]

Antibody-coated red cells are not always preferentially removed from the circulation, however, and a strongly positive direct Coombs' test (DCT) is compatible with normal red cell survival Such a situation is encountered relatively frequently in hypertensive patients treated with meth-

yldopa [5], it may also occur, for no identifiable reason in healthy subjects The frequency of this phenomenon in a donor population has been estimated at between 1 and 3 per 10,000 [6], and in at least one such subject red cell survival has been directly studied by $^{51}$Cr labelling and shown to be normal [7]

The present study was designed to compare the avidity with which DCT-positive red cells from actively haemolysing and non haemolysing subjects are engulfed by normal blood monocytes If red cells from the latter group are less readily phagocytosed it would suggest a difference in the type, quantity or configuration of erythrocyte-bound IgG between the two groups and thus explain the two different clinical pictures Equally active phagocytosis of red cells from each group, on the other hand, would imply either that this *in vitro* model is not a valid analogue of haemolysis *in vivo*, or alternatively that other characteristics, peculiar to the subjects themselves, distinguish the haemolysing from the non-haemolysing DCT positive individual

## *Subjects, Materials and Methods*

A total of 23 DCT positive subjects were studied They fell into four categories

*Cases of warm autoimmune haemolytic anaemia* [11] All of these patients were diagnosed by standard haematological criteria and manifested strongly positive direct Coombs tests with anti IgG reagents All but one of these patients were actively haemolysing when studied The exception had been in haematological remission and receiving no therapy for several years and is included in the non haemolysing category (fig 2)

*Babies suffering from Rhesus haemolytic disease* [3] Each of these showed evidence of accelerated haemolysis at the time of testing

*Hypertensive patients on methyldopa* [6] Methyldopa treated patients were screened regularly for evidence of red cell autoantibody formation and samples were obtained from those with strongly positive IgG direct Coombs tests

*Blood donors* [3] These subjects were found to have positive IgG direct Coombs tests in the course of routine serological testing Each of them were healthy and haematologically normal

*Red cell samples* Blood samples were stored as clotted or EDTA or ACD anti coagulated specimens for up to 5 days at 4 °C before testing Preliminary studies showed that samples stored in this way did not alter significantly in reactivity in the phagocytosis system Immediately before assay red cells were washed 3 times in normal saline and suspended at a haematocrit of 5% in Hanks solution containing 10% fetal calf serum

*IgG sensitised red cell controls* Aliquots of heparin anti coagulated red cells from the blood samples which provided normal monocytes for the same experiment

(see below) were washed 3 times in normal saline and incubated at 37 °C with equal volumes of dilutions of a standard Rhesus anti D antiserum This reagent comprised a pool of sera from four maternally iso immunised D negative women After 30 min incubation the red cells were again washed 3 times in normal saline and suspended in Hanks solution containing 10% fetal calf serum

*Monocyte phagocytosis assay* This technique is essentially identical to the method described by ABRAMSON *et al* [8] For each experiment venous blood was obtained from the same healthy normal subject and mixed with a half volume of 3% dextran in normal saline containing 10 units/ml of preservative free heparin After 1–1 5 h sedimentation at room temperature the white cells in the supernatant were washed 3 times in Hanks solution and suspended at a concentration of 6–8×10⁶/ml in Hanks solution containing 10% fetal calf serum Aliquots of the cell suspension (0 5 ml) were dispensed into 16 mm diameter flat bottomed plastic wells (Linbro Chemical Co Code FB54) After 1 h at room temperature during which time cells sedimented to the floors of the wells non adherent cells were removed by washing with two changes of Hanks solution Preliminary experiments established that the great majority of cells remaining after this step were monocytes and polymorphs The wells were then filled with 0.5 ml of red cell suspension each red cell sample being tested in duplicate The wells were sealed with parafilm and incubated at 37 °C for 2 75 h Non adherent red cells were removed by a single wash with Hanks solution followed by two washes in isotonic pH 7 4 buffered saline containing 2% bovine serum albumin The wells were air dried, fixed for 5 min in methanol and Giemsa stained The number of red cells engulfed by 200 monocytes was counted for each well and the mean of each duplicate calculated

*Calculation of strength of opsonisation* Red cells sensitised with IgG were avidly phagocytosed by monocytes under the conditions of these experiments and the degree of phagocytosis was directly related to the concentration of sensitising antiserum This relationship is illustrated in figure 1

Red cell samples from DCT positive subjects were tested in batches of between one and four at a time in the phagocytosis assay It was necessary to compensate for possible between experiment variations in the activity of monocyte monolayers prepared on different occasions This was achieved by including two IgG sensitised red cell controls in each experiment These controls consisted of red cells sensitised with two dilutions of the standard antiserum (0 125 and 0 03125) chosen because they had been shown to cause strong and weak phagocytosis, respectively (fig 1) The degree of phagocytosis of these two standards in any experiment was plotted on a log scale against the two serum dilutions, and a straight line drawn through these two points (giving a slope similar to that illustrated in figure 1) This graph was then used to convert phagocytosis figures for each DCT positive red cell sample tested in the same experiment into strength of opsonisation This value is the concentration of the standard antiserum which would have caused the same degree of phagocytosis if it were used to sensitise normal red cells for testing against the same monocyte monolayer

*Elution of antibody from direct Coombs positive red cells* The acid elution technique described by KIDD [9] was used for this purpose The red cells had been stored in ACD at 4 °C before elution

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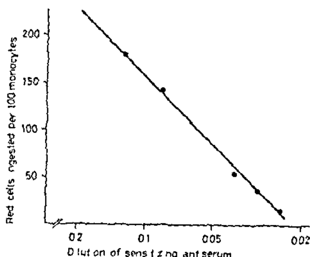



*Fig 1* Relationship between the dilutions of anti D antiserum with which D positive red cells are coated and the strength of phagocytosis by normal monocytes

*Autoanalyser testing of antibody potency* The agglutination of red cells in an autoanalyser circuit, in the presence of antibody, polyvinyl pyrolidone and bromelin was used to assay relative antibody potency [10 11]

## *Results*

*Phagocytosis of direct Coombs-positive red cells* The subjects who provided DCT-positive cell samples were divided into those in whom there was evidence of haemolysis (anaemia, reticulocytosis), and those who were haematologically normal The avidity with which red cells from the two groups were phagocytosed (strength of opsonisation) is shown in figure 2 It is apparent that red cells from non-haemolysing subjects were readily engulfed by monocytes (fig 3), and that the strength of phagocytosis in this group was no weaker than the reactions obtained with red cells from cases of immune haemolytic disease

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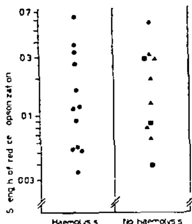



*Fig 2* The readiness with which red cells from haemolysing and non haemolysing subjects were engulfed by normal monocytes (see text for method of calculating 'strength of red cell opsonisation) ● = Haemolytic anaemia (active), ♦ = haemolytic anaemia (quiescent) ▲ = methyldopa patients, ■ = normal blood donors



*Fig 3* Red cells from a DCT positive healthy blood donor phagocytosed by normal monocytes Giemsa stain ×665

with four dilutions of the standard pooled anti D antiserum These sensitised cells were then tested in a single experiment with normal monocytes The eluate and standard antiserum were simultaneously tested against the same batch of red cells in the autoanalyser circuit

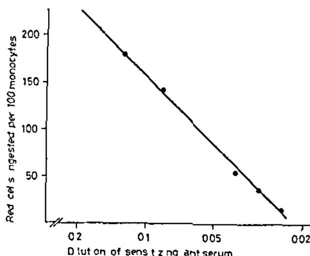



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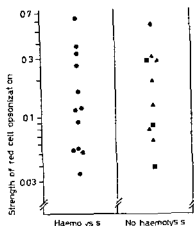



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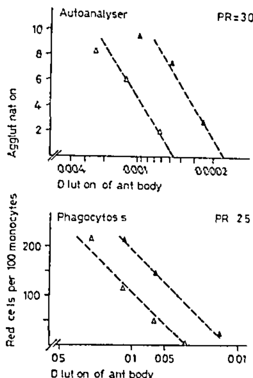



*Fig 4* The relative agglutinating (as measured in the autoanalyser circuit) and opsonising (as measured by monocyte phagocytosis) potency of an anti D antiserum compared with autoantibody eluted from the red cells of a DCT-positive blood donor PR = Potency ratio △ = Eluate, ▲ = anti-D

Figure 4 compares the relative potency of the two antibodies in the autoanalyser and phagocytosis system The eluate was approximately 2 5 times weaker than the anti-D antiserum in inducing phagocytosis and approximately 3 times weaker as an agglutinin

*Discussion*

The subclasses of IgG show considerable heterogeneity in their reactivity with the Fc receptors of mononuclear phagocytes Antibodies belonging to the classes IgG1 and IgG3 have been shown in a number of independent studies to bind considerably more strongly to Fc receptors than do IgG2 and IgG4 molecules [12–14] The fact that antibodies to the Rhesus-D antigen are predominantly in the IgG1 and IgG3 subclasses [15] appears to correlate well with the readiness with which these anti-

bodies cause accelerated red cell destruction *in vivo* Further support for this concept is found in the report by GELFAND *et al* [16] of a case of autoimmune haemolytic anaemia due to an IgG3 autoantibody in whom buffy coat erythro-phagocytosis could be demonstrated, and in the patient reported by GERGELY *et al* [17] with a positive direct Coombs' test due to a IgG4 antibody in whom there was no evidence of haemolysis ENGELFRIET [18] has studied two subjects whose red cells were coated with IgG2 autoantibody and a further individual with an IgG4 antibody In none of these cases was haemolysis demonstrable

Other findings, however, have raised the question of how closely the *in vitro* reactions of IgG antibodies reflect their haemolytic potential ENGELFRIET [18] reports that although IgG3 autoantibodies, in keeping with their strong binding to Fc receptors, almost always lead to accelerated red cell destruction, IgG1 red cell sensitisation on the other hand, which also produces striking phagocytosis *in vitro*, may be associated with normal red cell survival

The present results further support this concept that phagocytosis *in vitro* may not necessarily parallel the fate of red cells *in vivo*, in that erythrocytes from non-haemolysing subjects were readily ingested by normal monocytes In addition it was found that antibody eluted from the red cells of a DCT-positive healthy blood donor was as potent an opsonin *in vitro*, as it was an agglutinin If the strength of agglutination in the autoanalyser circuit is proportional to the number of IgG molecules carried per red cell, the latter experiment suggests that, molecule for molecule, 'harmless' auto-antibodies found in healthy subjects are as avidly bound by mononuclear cell Fc receptors as are haemolytic antibodies

The absence of haemolysis in DCT-positive subjects is explicable in several ways It may represent hitherto undiscovered characteristics of their IgG-coated red cells These features may be intrinsic to the antibodies themselves, or possibly involve accessory phenomena, such as the binding of additional factors to the red cell surface, or peculiarities of the erythrocyte membrane Whatever the characteristics of the antibody-coated cells which allow them to escape haemolysis, however, the monocyte phagocytosis assay appears not to recognise them

An alternative explanation is that these subjects fail to recognise and destroy IgG-sensitised red cells because of some abnormality in their phagocytosis mechanisms Survival studies of transfused IgG-sensitised red cells from these subjects in normal recipients could answer this question, although rapid elution of antibody when the cells enter the new envi-

ronment might possibly give spuriously prolonged survival Preliminary experiments using the monocyte assay have shown that monocytes from two of the blood donors in this series were *fully* capable of ingesting IgG-sensitised red cells, but this does not preclude abnormalities of recognition and phagocytosis in the spleen and liver in these individuals

*Acknowledgements* I am grateful to Mr P BOWELL, Dr M FISHER and Dr J GRANT for providing DCT-positive samples from normal blood donors and for the Rhesus antiserum, to Miss N JONES for samples from DCT positive patients, to Dr J MORTON who kindly assayed samples in the autoanalyser circuit, to Dr T PARRY who kindly photographed the figures, and to Dr M M PICKLES in whose laboratory this work was carried out

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Acta haemat 55 10–20 (1976)

# Erythrocyte Enzymes in Neonatal Jaundice[1]

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Department of Obstetrics and Gynaecology University College Hospital Ibadan

*Key Words* Erythrocyte enzymes Glucose-6 phosphate dehydrogenase Glutathione peroxidase Glutathione reductase Neonatal jaundice

*Abstract* Glucose 6-phosphate dehydrogenase (G6PD) deficiency is a major cause of severe neonatal jaundice in Nigeria but not all G6PD-deficient babies become jaundiced Neonatal jaundice not attributable to G6PD deficiency nor to any other known aetiology is also common In an effort to explain these two facts, we have measured the levels of the three enzymes G6PD glutathione peroxidase (GSHPX) and glutathione reductase (GSSGR) in 38 jaundiced newborns, 26 control newborns and 44 normal adults all of them males We could not yet prove an additive effect of GSSGR or GSHPX deficiency with G6PD deficiency in causing jaundice There was no evidence that low levels of GSHPX *per se* are associated with jaundice However jaundiced newborns with normal G6PD had significantly lower levels of GSSGR than control newborns with normal G6PD These data suggest that a relatively low activity of GSSGR a riboflavin-dependent enzyme, may predispose the red cells to accelerated destruction in the neonatal period

Erythrocytes of newborns are generally more susceptible to haemolysis than those of adults and it has been proposed that hydrogen peroxide may be an important cause of oxidative damage to these cells [2] At least three enzymes are known to be involved in the detoxification of $H_2O_2$ in the erythrocyte GSHPX[2], which directly destroys $H_2O_2$ GSSGR which

[1] This work received financial support from USPHS grant GM 17261 and from the World ... am ... [BIE]NZLE was a Research Fellow sponsored by the Deutsc[h] ...

[2] The f... used ACD = a... dextrose FAD = fla... reduced glutath... = oxi... dized glutathi... GSSGR ... redu... tase G6P = ... phosph... nase NADP = ni... N... uced NADP 6PG = ...

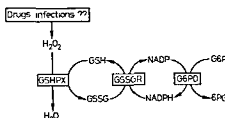



*Fig 1* Main pathway of detoxification of hydrogen peroxide in human erythrocytes The disposal of $H_2O_2$ by the alternative catalase reaction is likely to be relatively ineffective at the expected intracellular concentration of $H_2O_2$ [5], especially in newborns who have in general catalase levels lower than adults [22]

regenerates the reduced glutathione consumed by GSHPX, and G6PD, which regenerates the NADPH consumed by GSSGR (fig 1) One could therefore expect that deficiency or decreased activity of one or more of those enzymes might lead to hyperbilirubinaemia in the newborn Indeed, genetically determined G6PD deficiency has been identified as an important cause of neonatal jaundice in Greece since 1961 [6] Genetically determined deficiency of GSHPX has also been associated with neonatal jaundice in some cases [20], and newborns with low levels of this enzyme may have an increased chance of developing severe degrees of hyperbilirubinaemia [30] Regarding GSSGR and its possible importance as an aetiological factor in the pathogenesis of neonatal jaundice, there is no information as yet in the literature to the best of our knowledge

In a preceding paper from this institution [8] the high prevalence of neonatal jaundice in Southern Nigeria has been documented, and G6PD deficiency has emerged as one of the major aetiological factors In this study we have specifically tested the possible role of the other two enzymes mentioned GSHPX and GSSGR, in the pathogenesis of hyperbilirubinaemia in the newborn, and their possible interactions with G6PD itself It will be shown that low levels of GSSGR correlate significantly with the development of jaundice

## *Subjects and Methods*

G6PD GSHPX and GSSGR were tested in three groups of subjects (1) 38 male newborns 3 of them prematures with birth weights below 2 500 g, admitted to the

[illegible] Hospital Ibadan because of jaundice [illegible], (2) 26 normal male newborns de [illegible] group of 44 adult blood donors

[illegible] a combination of the following tech [illegible] to PORTER *et al* [24] with slight modifi [illegible] according to the recommendations of WHO [illegible] [11]

[illegible] *to* PAGLIA and VALENTINE [23] *Blood* [illegible] washes with cold isotonic NaCl solution the [illegible] deionised $H_2O$ 0 005 ml of haemolysate [illegible] strength Drabkin solution was added to [illegible] of 2.5 ml of 0 05 M phosphate buffer pH 7 1 [illegible] EDTA [illegible] NADPH 0 1 ml of 0 15 M GSH 0 01 ml [illegible] purified GSSGR (Boehringer) After 5 min [illegible] *the reaction was started by adding 0 02 ml of* [illegible] and the change of optical density at 340 nm was fol [illegible] Gilford multisample absorbance recorder At the same time [illegible] of GSH was measured by adding the same amount of $H_2O_2$ [illegible] of haemolysate and the resulting slope was subtracted from that of the enzymatic reaction

GSSGR activity was measured according to HORN [15] The components of the assay system were always added in the following sequence 0 7 ml of 0 067 M phos phate buffer pH 6.6 0.2 ml of 7.5 mM GSSG 0 05 [illegible] 6 mM NADPH and the reaction was started by addition of 0 05 ml of hae [illegible] In a number of cases haemolysates of newborns were incubated for 1 [illegible] emperature with FAD to give a final concentration of 7 μM befo [illegible] SSGR was per formed

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*Table I* High prevalence of G6PD deficiency in babies with neonatal jaundice in Nigeria

| | G6PD deficiency, % |
|---|---|
| Normal adults [17] | 21 6 |
| Normal newborns [NYLANDER and LUZZATTO, unpublished results] | 22 5 |
| Newborns with neonatal jaundice [8] | 51 0 |
| Newborns with neonatal jaundice [this study] | 45 0 |
| Newborns with severe neonatal jaundice (serum bilirubin >18 mg/100 ml) [this study] | 78 5 |

*Table II* Statistical analysis of differences in levels of GSSGR

| G6PD status | Comparison | p level |
|---|---|---|
| Normal | normal newborns *vs* adults | <0 01 |
| Deficient | jaundiced newborns *vs* normal newborns | >0 10 |
| Normal | jaundiced newborns *vs* normal newborns | <0 02 |

*Table III* Effect of FAD on GSSGR activity in haemolysates from newborns

| G6PD status | GSSGR activity, IU/g Hb (mean ± SD) | | Stimulation, % |
|---|---|---|---|
| | no addition | +7 μM FAD | |
| Normal (n = 7) | 12 0 ± 2 3 | 23 0 ± 4 5 | 92 |
| Deficient (n = 5) | 19 8 ± 2 9 | 26 5 ± 3 7 | 35 |

and 2 were G6PD normal) (table I) The proportions are in good agreement with the data reported by EFFIONG *et al* [8] on a larger series by a screening method alone Electrophoretic characterization of the enzyme from G6PD normal babies showed that 15 had type B and 6 had type A, in agreement with the distribution of G6PD variants observed by LUZZATTO and ALLAN [17] in adults, and by NYLANDER and LUZZATTO [unpublished results] in newborns (G6PD type B 55 5%, type A 22 9%, type A⁻ 21 6%)

*Glutathione peroxidase* A wide scatter in the values of activity was observed in all three groups studied (fig 2) GSHPX levels are signifi-

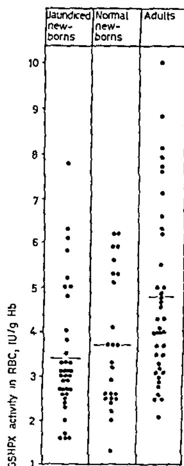



*Fig 2* GSHPX levels in the three groups of subjects studied Each dot represents one subject Horizontal bars represent arithmetical means

cantly higher in adults than in newborns with or without jaundice, but there is extensive overlap in the distribution The average activity of GSHPX is lower in babies with neonatal jaundice than in normal newborns, but the difference between these two groups does not reach significance *The distribution of enzyme activities of G6PD-normal and G6PD-deficient subjects does not differ significantly if each group is considered separately*

*Glutathione reductase* Since GSSGR is increased in G6PD deficiency [27], the data on this enzyme are presented separately for G6PD-normal and G6PD-deficient subjects (fig 3) As expected, in all three groups G6PD-deficient subjects have significantly higher GSSGR levels than

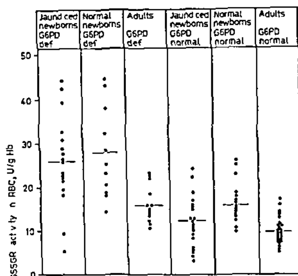



*Fig 3* GSSGR levels in the three groups of subjects studied subdivided according to G6PD status Each dot represents one subject Horizontal bars represent arithmetical means

G6PD normal individuals The average GSSGR values were significantly higher in both groups of newborns than in adults, as has been previously demonstrated for premature and normal babies by GROSS *et al* [13] When jaundiced and normal newborns are compared, the former are found to have lower GSSGR activities The difference does not reach significance in G6PD deficient newborns, but it is significant in G6PD normal babies (table II) No significant correlation was found between gestational age, birth weight and GSSGR and GSHPX activity in G6PD-normal and G6PD-deficient babies with and without jaundice We also could not detect any relationship between the clinical picture, serum bilirubin levels and enzyme activities in the patients with neonatal jaundice

*Stimulation of GSSGR by FAD* The coenzyme of GSSGR is FAD, and dietary intake of riboflavin or addition of FAD in minute amount to haemolysates increases the activity of GSSGR [1] In 13 newborns (with or without jaundice) haemolysates were assayed for GSSGR before and after preincubation with FAD The percent increase in GSSGR activity is much higher in haemolysates with low initial GSSGR activity (table III) Thus, after addition of FAD, the absolute GSSGR activity is very similar

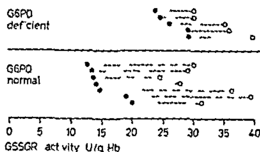



*Fig 4* Effect of 7 µM FAD on GSSGR activity of crude haemolysates See data of table III For each subject the full circle represents the activity without added FAD and the open circle connected to the full circle by a broken line represents the activity for the same subject with added FAD

in the two groups of haemolysates It appears that G6PD deficient red cells, rather than having more GSSGR, have GSSGR which is already nearly saturated with FAD (fig 4) [19]

## *Discussion*

EFFIONG *et al* [8] have demonstrated the major role of G6PD deficiency in the pathogenesis of neonatal jaundice in Nigeria, in agreement with previous reports on smaller series [12, 14, 25] Since we found that all of the G6PD deficient jaundiced newborns had the A⁻ type of this enzyme, this variant must be included, together with the Mediterranean variant [6] and with those encountered in the Far East [19] amongst those which increase the risk of clinically significant haemolysis in the perinatal period EFFIONG *et al* [8] also pointed out that, apart from ABO incompatibility (less than 20% of our patients), the pathogenesis of neonatal jaundice remains obscure in a substantial proportion of cases In the present study we were therefore concerned mainly with two specific questions

(1) Since not all G6PD deficient babies develop jaundice, what is the additional factor, or factors responsible for this complication? It appeared possible that the jaundiced babies might also have, in addition to genetically determined G6PD deficiency, perhaps transiently and on a non genetic basis a low level of either GSSGR or GSHPX Indeed this was found to be true for both enzymes, but with the number of cases presently available, the differences observed are not statistically significant

Thus, the notion that the additive effect of two enzyme abnormalities might be a cause of neonatal jaundice, remains a hypothesis which we are continuing to test

(2) Since GSSGR and GSHPX, in addition to G6PD, are both involved in detoxification of hydrogen peroxide, it is possible that low levels of either or both of these enzymes are associated with the development of otherwise unexplained neonatal jaundice As far as GSHPX is concerned, although NECHELES *et al* [20] have reported association of partial deficiency of this enzyme with haemolytic disease in neonates, we could not find evidence so far that this plays a role in the pathogenesis of neonatal jaundice in Nigeria We also could not demonstrate any correlation between GSHPX and GSSGR activity in individuals of any of the three groups studied On the other hand, while there does not seem to be any reference to the possible role of GSSGR in the development of neonatal haemolysis, our data show clearly that the levels of this enzyme in jaundiced babies are lower than in normal babies, once both groups are matched with respect to their G6PD status On theoretical grounds, this finding is not surprising, since a number of workers [3, 4, 10, 16] have already suggested that low levels of GSSGR can be associated with clinically important haemolysis

Based on the number of cases presently available, the difference in GSSGR levels is not statistically significant amongst G6PD deficient babies, but it is significant at the 2% level in the G6PD normal babies Since G6PD deficient subjects have, as a rule, higher GSSGR, it is conceivable that these two enzymes interact in a peculier way with respect to causing neonatal jaundice paradoxically, G6PD deficiency might protect a baby against developing a dangerously low level of GSSGR It is not likely that low GSSGR is an effect, rather than a cause, of neonatal jaundice, since the activity of this enzyme is higher in young that in old fetal erythrocytes [29], and haemolysis would therefore, if anything, raise its level in whole blood

These results suggest that GSSGR may be important in the pathogenesis of severe neonatal jaundice in Nigeria The incidence of 'deficiency' of this enzyme will have to be established by testing an unselected group of newborns However, it would not be surprising if it was relatively common, since it is now known to occur rarely, if ever, on a genetic basis, rather, it usually results from a low dietary intake of riboflavin [7], and suboptimal riboflavin levels are prevalent in Southern Nigeria For instance, 37% of a group of pregnant women exhibited very low urinary ri-

boflavin excretion values consistent with vitamin deficiency [21] The ratio of activity of GSSGR with and without added FAD is itself a sensitive index of the riboflavin status in man [26 28] and the fact that the FAD stimulation observed by us (fig 4) is much more marked than reported on cord bloods in USA [32] is consistent with the possibility that many of our newborns have suboptimal levels of riboflavin Treatment with riboflavin of GSSGR-'deficient' subjects increases the enzyme activity to normal or above normal levels [1 28] We can thus entertain the contention that administration of riboflavin to pregnant women near term may help to prevent neonatal jaundice in at least some cases in this community

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# Blood Coagulation in Patients with Acute Infectious Hepatitis in India

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*Key Words* Bleeding disorders Blood coagulation Hepatitis Liver diseases

*Abstract* Coagulation studies were performed in 61 patients of acute infective hepatitis 18 with clinical signs of liver failure had bleeding and all succumbed The 47 patients without liver failure showed no haemorrhagic diathesis and all of them had uneventful recovery Though coagulopathy was present in most of the patients the severity and frequency of coagulation defects were more in those with signs of hepatic failure Hypofibrinogenemia, elevated serum fibrinogen degradation products and accelerated euglobulin lysis were conspicuous in patients with hepatic failure It appears that while diminished synthesis of coagulation factors is the main basis for coagulopathy in patients without hepatic failure, additional factors like local or disseminated intravascular coagulation and increased fibrinolysis also contribute significantly to the coagulopathy in cases of liver failure

Blood coagulation abnormalities in liver disorders are frequent since liver plays important roles in synthesis of coagulation factors [11] and in clearance of activated clotting factors [5] and plasminogen activator [7] from the circulation However, though many studies on chronic liver disease particularly hepatic cirrhosis are available [6, 17] observations on acute hepatocellular disease are conspicuously few Only recently an exhaustive study has appeared from Australia [8] The present paper describes our observations on the coagulation profile of 61 patients of infective hepatitis in India

## *Material and Methods*

The 61 patients included in this series were either in patients of Medical, Obstetrics and Gynaecology and Student Wards, or out patients from Students' Clinic of

SSL Hospital Banaras Hindu University They were investigated during an epidemic outbreak of *infective hepatitis during the winters of 1972–73* The diagnosis was based on clinical history, physical examination and corroborated by liver function tests The patients were divided into two groups (1) 43 patients (34 males and 9 females) without signs of liver failure and (2) 18 patients (14 females and 4 males) with signs of liver failure, as determined by mental changes, flapping tremor and fe tor hepaticus [8] 12 of them were in coma grade IV and the remaining 6 in coma grade III [18] *The patients were investigated within 1–2 weeks of clinical appear* ance of jaundice, the serum bilirubin *ranging* between 2 and 15 *mg% at the time of* investigation

Routine haematological tests were done as described by Dacie and Lewis [2] Coagulation tests were mostly done as described by Denson [3] The other tech niques used were plasma fibrinogen [13] euglobulin lysis time [1] Astrup fibrin *plate lysis as described by Dacie and Lewis [2] using bovine fibrinogen (Sigma)* thrombin time [12] serum FDP [10] and PF 3 availability [9]

## *Results*

Clinical haemorrhagic diathesis in these patients was conspicuously re- lated with signs of hepatic failure All the patients with liver failure had clinical bleeding (table I) and all ultimately succumbed to the disease, while none of the patients without signs of liver failure showed clinical bleeding diathesis The latter group of patients uniformly had uneventful recovery Blood coagulation disturbances, however, occurred irrespective of the signs of hepatic failure (table II) Statistical analysis indicated sig- nificant prolongation of kaolin cephalin clotting time (KCCT), pro- *thrombin time (PT) and thrombin time (TT) in both groups of patients*

*Table I* Bleeding symptoms in infective hepatitis patients with hepatic failure

| Bleeding symptoms | Females (n = 14) | Males (n = 4) |
|---|---|---|
| Petechiae | 1 | 0 |
| Ecchymoses | 2 | 1 |
| Bleeding from gums | 2 | 0 |
| Haematuria | 4 | 0 |
| Haematemesis | 9 | 4 |
| Post partum haemorrhage | 6 | – |
| Ante-partum haemorrhage | 3 | – |

Factor II, V and VII+X were assayed in 18 patients without hepatic failure These factors were reduced in 40, 57 and 61% of patients respectively The lowest value of factor II was 25% in one patient Factors V and VII+X were less than 10% in 4 patients (22%)

The platelet changes were of a mild nature only, the lowest platelet count recorded was 120,000/μl in one case Platelet factor 3 (PF 3) availability index of less than 25% was noted in 4 patients, all of whom had hepatic failure Platelet aggregation was grossly abnormal in one patient with liver failure where the platelets completely failed to aggregate with adenosine diphosphate and epinephrine and where no PF 3 activation occurred with kaoline Statistically, the reduction of platelet count was significant in only those with hepatic failure but PF 3 availability index was significantly low in both groups of patients Plasma fibrinogen was not significantly altered in patients without liver failure In contrast, half of the patients with liver failure had plasma fibrinogen levels less than 150 mg% Statistically also there was significant hypofibrinogenaemia in this group Significant increase of fibrinolytic activity was detected in these patients The patients with and without liver failure had mean euglobulin lysis time (ELT) of 155 and 200 min, respectively (control 220 min), and the respective mean figures for the lysis zone on the Astrup fibrin plate caused by euglobulin fraction were 40 and 33 mm² (control 18 mm²) Serum fibrinogen degradation products (FDP) were statistically elevated to a significant degree in both groups of patients, however, levels more than 20 μg/ml were only noted in 9 patients with hepatic failure Statistical analysis showed that all the tests, except Astrup plate lysis, were significantly more deranged in patients with hepatic failure as compared to patients without hepatic failure

## *Discussion*

The high proportion of female patients with hepatic failure in this series is partly due to the treatment of uncomplicated female patients as out patients who were not investigated Secondly, there appears to be a true increase of susceptibility of pregnant patients to develop hepatic failure

The majority of the patients with acute infective hepatitis had blood coagulation abnormalities of varying degree The defects were more pronounced and more frequent in cases with liver failure as compared to those

*Table II* Coagulation parameters in infective hepatitis

| | Kaolin cephalin clotting time, sec | Prothrombin time, ratio | Thrombin time, ratio | Platelets $\times 10^3/\mu l$ |
|---|---|---|---|---|
| Group I patients without liver failure | | | | |
| Range | 35–177 | 1.0–2.3 | 1.0–2.0 | 120–400 |
| Mean±SD | 76±27** | 1.3±0.34** | 1.3±0.26** | 260±50 |
| | (n=43) | (n=43) | (n=24) | (n=43) |
| Group II patients with liver failure | | | | |
| Range | 67–*a* | 1.1–*a* | 1.1–*a* | 160–350 |
| Mean±SD | 145±44**++ | 2.4±2.05**++ | 2.3±0.83**+++ | 220±40*++ |
| | (n=18) | (n=18) | (n=18) | (n=18) |
| Control | | | | |
| Range | 40–65 | – | – | 220–500 |
| Mean±SD | 56±10 | | | 264±75 |
| | (n=75) | | | (n=19) |

* Mark indicates the statistical comparison of values with control
\+ Mark indicates the statistical comparison of values with group I

without liver failure. Clinically too the bleeding was manifest in patients with liver failure only. These observations are in complete agreement with the experience of GALLUS *et al.* [8].

Thrombocytopenia was not an important feature in patients with infective hepatitis. Only in 3 out of 61 cases, platelet counts were 150,000/$\mu$l or less. Platelet aggregation with ADP and PF 3 activity too did not exhibit any remarkable abnormality in patients without liver failure. PF 3 availability was significantly poor (less than 25% availability index) in 4 patients, all with liver failure.

The patients without liver failure showed lesser degree and frequency of prolongation of prothrombin time, KCCT and thrombin time. Factors II, V and VII+X estimated in 18 patients showed corresponding decreases in 40, 57 and 61% of cases respectively. Previous studies in infective

*Table II* (continued)

| PF 3, % | Factor I mg% | Serum FDP µg/ml | Euglobulin lysis time min | Fibrin plate lysis, mm² |
|---|---|---|---|---|
| 25 100<br>72±25 5**<br>(n=23) | 168-600<br>302±103<br>(n=43) | 2 5–20<br>7±4 8**<br>(n=23) | 60-360<br>200±72 4*<br>(n=43) | 16–64<br>33±11 5**<br>(n=23) |
| 0–100<br>40±22 8**++<br>(n=14) | 90–375<br>199±103**++<br>(n=18) | 5–80<br>38±33 7**++<br>(n−18) | 76–240<br>155±45 6**++<br>(n=18) | 16–56<br>40±11 2**<br>(n=14) |
| – | 270–510<br>304±84 7<br>(n=19) | 0–5<br>1 6±2 25<br>(n=10) | 180–375<br>220±48<br>(n=20) | 0–42<br>18±10 8<br>(n=15) |

* and + p=0 05, ** and ++ p−0 01, +++ p=0 001

hepatitis [4, 8, 16] showed a similar reduction of these coagulation factors Plasma fibrinogen was mildly lower (150–200 mg%) in only 5 (11 5%) cases Conspicuous increase of fibrinolytic activity was notable only in a small percentage of patients, i e , euglobulin lysis time less than 2 h was seen in 5 cases and Astrup fibrin plate lysis zone more than 50 mm² occurred in only 2 patients FDP were not beyond 10 µg/ml in any patient The prolongation of prothrombin time and reduction of coagulation factors II, V and VII+X in patients without liver failure would appear, therefore, to be resulting mostly from the diminished synthesis of these factors in the damaged liver

The blood coagulation disturbances were uniformly more severe in patients with liver failure All of them had prolonged prothrombin time and KCCT and about two thirds of them showed hypofibrinogenaemia 28%

of them showed definite acceleration of plasma euglobulin lysis and 50% showed elevated levels of serum FDP GALLUS *et al* [8] observed greatly reduced plasminogen levels in most patients and lowered plasma fibrinogen in over half of their patients RAKE *et al* [14] reported reduced fibrinogen survival in patients with viral hepatitis and liver failure and attributed it to disseminated intravascular coagulation In another paper RAKE *et al* [15] reported highly encouraging results with early administration of heparin in patients with liver failure It appears that the pathogenesis of blood coagulation defects in these cases (with liver failure) is multifactorial, diminished hepatic synthesis of coagulation factors elevated fibrinolytic activity and local/disseminated intravascular coagulation playing the contributory role to varying extent

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of them showed definite acceleration of plasma euglobulin lysis and 50% showed elevated levels of serum FDP. GALLUS *et al.* [8] observed greatly reduced plasminogen levels in most patients and lowered plasma fibrinogen in over half of their patients. RAKE *et al.* [14] reported reduced fibrinogen survival in patients with viral hepatitis and liver failure and attributed it to disseminated intravascular coagulation. In another paper RAKE *et al.* [15] reported highly encouraging results with early administration of heparin in patients with liver failure. It appears that the pathogenesis of blood coagulation defects in these cases (with liver failure) is multifactorial, diminished hepatic synthesis of coagulation factors, elevated fibrinolytic activity and local/disseminated intravascular coagulation playing the contributory role to varying extent.

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# Involvement of the Central Nervous System in Rats with Acute Leukemia L 5222[1]

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*Key Words* Central nervous system hemorrhages Leukemia Meningeal leukemic infiltration Rat leukemia L 5222

*Abstract* Young adult BD IX rats made leukemic by intravenous infusion of $2 \times 10^7$ nucleated blood cells from leukemic donors (L 5222) showed lesions in the central nervous system 6 days after transfer when their leukocyte count was between 250 000 and 350 000 cells/μl The brain was affected in only half of the animals whereas all showed an infiltration of the meninges Furthermore all animals had extensive lesions in the spinal cord The lesions consisted of nodules of leukemic cells and hemorrhages which were predominant in the lower half of the spinal cord and more abundant in the white than in the gray matter Paraplegia appeared in some animals shortly before death Since there is a consistent involvement of the meninges and spinal cord the L 5222 leukemia seems to be a useful model for the study of leukemic infiltration of the central nervous system and the reaction to chemotherapy

Leukemic involvement of the central nervous system particularly of the meninges, constitutes a neurological complication in human acute leukemia During a study of the growth characteristics of a transplantable acute leukemia in rats (L 5222) carried out in our laboratory, some of the animals developed symptoms of involvement of the central nervous system that might have contributed to their death The present report describes the location, extension and microscopic characteristics of the invasion of the nervous system by leukemic cells in a group of rats in which the brain and spinal cord were systematically examined

[1] Research supported by Euratom Contract No 098 72 1 BIAC (Art No BIO 1095) and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 112 Projekt B2)

## Material and Methods

Six male rats of the inbred strain BD IX [1] weighing about 200 g were transfused with $2 \times 10^7$ leukocytes obtained from the blood of a leukemic donor of the same strain The cells had been frozen, stored under liquid nitrogen and thawed immediately before transfusion Details of the transfer and storage procedures were given by HOELZER [2]

The acute rat leukemia L 5222 used in this experiment was chemically induced with ethylnitrosourea by IVANKOVIC and ZELLER [3] who found no evidence for virus infection by means of the Gross technique Based on cytochemical characteristics the leukemia was classified as of immature monocytic type with a myeloid component [3]

All animals were sacrificed 6 days after transfer of the leukemic cells A complete autopsy was performed and blocks of tissue from brain and spinal cord were fixed in formalin and imbedded in paraffin for histological studies Shortly before sacrifice leukocyte counts and smears were made from tail vein blood The smears were stained with the May-Grunwald Giemsa method

## Results

Six days after intravenous injection of $2 \times 10^7$ leukemic cells all the animals had a high number of leukocytes in peripheral blood, reaching values between 265 000 and 364,000/$\mu$l One rat was paraplegic

The location and severity of the lesions found in the central nervous system are given in table 1 The cerebrum had no lesions in three animals and showed several small hermorrhages in three other rats The hemorrhagic areas contained also small groups of leukemic cells (LC) The cortex was invaded in only one rat The diencephalon showed lesions in three rats, without preference for any particular structure in that area The cerebellum had a few small hemorrhages with LC in four rats and was normal in two animals The meninges were diffusely infiltrated with a small number of LC in all animals

Severe lesions were found in the spinal cord (fig 1) All animals showed abundant LC in the epidural space spread along most of the spinal canal Four animals had numerous hemorrhages and masses of LC, mostly in the white matter of the spinal cord Figure 2 illustrates the location and size of the lesions in the lumbar region of one of the rats The gray matter appeared less affected, only some areas of the dorsal column, adjacent to the hemorrhages of the white matter, were damaged

The vertebrae were extensively infiltrated with LC In some areas groups of LC burst through Haversian canals into the spinal canal and

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*Fig 3* Vertebra (V) and spinal cord (SC) of rat 5 showing numerous hemorrhagic lesions in the white matter 6 days after intravenous transfusion of $2 \times 10^7$ leukocytes obtained from a donor animal of the same strain × 9

spread over the dura mater along the epidural space (fig 3) Some spinal ganglia were surrounded by numerous LC The leptomeninges in the brain were diffusely infiltrated as illustrated in figure 4

*Discussion*

Involvement of the central nervous system in rats with leukemia L5222 is more frequent than one may suspect by observing only neurological symptoms All animals in our series had infiltration of LC in the meninges and four out of six had hemorrhages and masses of LC in several areas of the nervous system, but only one had paraplegia The main lesions were found in the lower thoracic and lumbar segments of the spinal cord Lesions in these areas are responsible for paralysis of the hind legs Extensive spread of LC in the epidural space seems to be due to invasion of LC originating in the bone marrow of the vertebrae, where they settle and proliferate shortly after transfusion [4] and communicate with the spinal canal through Haversian canals of the vertebrae

*Table I* Location and severity of the lesions found in the central nervous system of rats with leukaemia L 5222

| Rat No | Leuko-cytes/µl | Brain | | | Spinal cord | | |
|---|---|---|---|---|---|---|---|
| | | cerebrum | cerebellum | meninges | cervical | thoracic | lumbar |
| 1 | 265,800 | (-) | (-) | LC (+) | LC epidural (+) | LC epidural (+) | LC epidural (+) |
| 2 | 348,800 | (-) | (-) | LC (+) | LC epidural (+) H and LC in white matter (+) | LC epidural (+) | LC epidural (+) |
| 3 | 364,000 | H and LC in dorsal cortex and corpus callosum (+) | H and LC in stratum granulosum of vermis (+) | LC (++) | LC epidural (+) | LC epidural (+) | LC epidural (+) |
| 4 | 309,000 | (-) | H and LC in molecular layer of vermis (+) | LC (+) | (-) | LC epidural (+) | LC epidural (+) H and LC in white matter (+) |
| 5 paraplegic | 327,000 | H and LC in diencephalon (++) | H and LC in white matter of vermis (+) | LC (++) | LC epidural (+) | LC epidural (+) white matter (+) gray matter (+) H and LC (+) | LC epidural (++) H and LC in white matter (++) gray matter (+) |
| 6 | 303,000 | H and LC in diencephalon (+) | H and LC in white matter of vermis (+) | LC (+) | (-) | (-) | LC epidural (+) H and LC in the white matter (+) |

H = Hemorrhage, LC = leukemic cell infiltraton, (++) = numerous and large, (+) = few and small, (-) = no lesions




Fig 3 Section of spinal cord (SC) and lumbar vertebra (V) The epidural space (arrow) is infiltrated with leukemic cells The white matter shows two small masses of leukemic cells (LC) and extensive hemorrhage (H) × 125

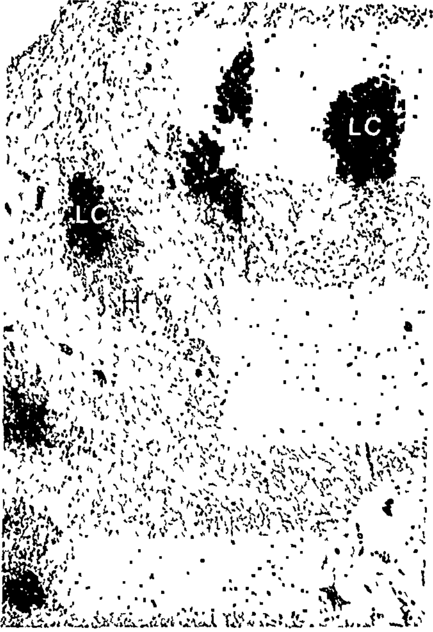



*Fig 2* Section of spinal cord stained with hematoxylin and eosin Masses of leukemic cells (LC) and associated hemorrhages (H) in the white matter of the lumbar region × 175



*Fig 4* Diffuse infiltration of leukemic cells in the leptomeninges of the brain (arrow) × 500

Predominance of the lesions in the white matter may be explained by peculiarities of the vascular pattern of the spinal cord in which the nutrient vessels reach the gray matter after traversing the white matter Embolisms occasioned by the abundant large cells circulating in the blood are likely to occur more often in areas first reached by LC The cells trapped in the narrow vessels continue their proliferation and distend the vessel wall to its rupture point That such a destruction can occur has previously been demonstrated for the sinus endothelial cells of the bone marrow [5]

A contributing factor to the production of hemorrhages may be the progressive diminution of platelets in the blood of the leukemic rats In a group of animals investigated under the same conditions as described here [6] it was found that the number of platelets declined to about one tenth of normal value 6 days after transfusion of LC The bleeding may be further due to disorders of hemostasis known to develop during the course of this leukemia [7]

Involvement of the central nervous system and of the meninges in particular is a known complicating factor in human acute leukemia especial

ly in children Since involvement of the meninges is a consistent finding in the experimental L 5222 leukemia, and further since remissions can be induced in this leukemia by chemotherapy [8] it seems to be a suitable model for the study of leukemic infiltration of the central nervous system and its reaction to chemotherapy

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Involvement of the central nervous system and of the meninges in particular is a known complicating factor in human acute leukemia, especial-

*Table I* Hb Barts in 650 newborns

| Amount of Hb Barts, % | Number of cases | Percent total | Percent with Hb Barts |
|---|---|---|---|
| 0 | 621 | 95 54 | 0 |
| 1–2 | 21 | 3 23 | 72.4 |
| 5 | 7 | 1 08 | 24 2 |
| 25 | 1 | 0 15 | 3 4 |
| Total | 650 | | |

determined in Cuba [1], no data are available on the incidence of $\alpha$-thalassaemia We report here a study carried out to estimate the frequency of this entity

## *Materials and Methods*

650 normal full term newborns were chosen at random from the Nursery of the E Cabrera Hospital in Habana Capillary blood was collected in heparinized tubes Haemoglobin types were identified by starch gel electrophoresis using the Tris-EDTA borate buffer of SMITHIES [7] at pH 8 6 Hb Barts was quantitated by cellulose acetate electrophoresis in the same buffer

In all cases showing high Hb Barts, a haematological study of the family was done, including serum iron determination

## *Results*

Hb Barts was detected in 29 out of 650 newborns, which gives an incidence of 4 46% The amounts of Hb Barts were segregated into 3 groups showing 1–2, 5 and 25%, respectively The data are summarized in table I

## *Discussion*

High percentages of Hb Barts are usually considered characteristic of $\alpha$-thalassaemia and its levels are positively correlated with the severity of the disease [6] Moreover, the trimodal distribution of this haemoglobin found in newborns (not including the cases of hydrops fetalis, which would constitute a fourth group) allows a genetical interpretation of the $\alpha$-thalassaemias, and also permits to anticipate the type of thalassaemia

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# $\alpha$-Thalassaemia in Cuba

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*Key Words* $\alpha$ Thalassaemia Hb Barts Haemoglobinopathies

*Abstract* The frequency of $\alpha$ thalassaemia has been determined by the analysis of Hb Barts in 650 newborns, 4 46% of them showed high levels of Hb Barts The incidence of newborns with different levels of Hb Barts (1–2, 5 and 25% respectively) and the frequency of the $\alpha$ thalassaemia genes are also given

The $\alpha$-thalassaemia syndromes are a group of hereditary microcytic anaemias of different severity, characterized by variable reductions in the rate of synthesis of the $\alpha$-globin chain [11] Genetical and clinical evidences indicate that there is more than one type of $\alpha$-thalassaemia, but the genetical lesion(s) responsible for these entities is not fully understood Recently it has been demonstrated that a deletion of the $\alpha$-gene(s) produces the homozygous $\alpha$-thalassaemia, which results in intrauterine or early neonatal death with a clinical picture of hydrops fetalis, associated with large amounts of haemoglobin Barts (Hb Barts) [5, 9] On the other hand, only mild haematological changes characterize the $\alpha$-thalassaemia trait [11] Its identification is therefore extremely difficult in the adult, whereas the presence of excess $\gamma$-chains in neonates makes it possible to recognize the $\alpha$-thalassaemia trait by the quantitation of Hb Barts ($\gamma_4$) during the newborn period [3] With this method the incidence of $\alpha$-thalassaemia has been determined in different countries, it has been found to be very frequent in South East Asia, where comprehensive investigations have been carried out [3, 4], and to be present also in the Mediterranean area and in African and American Negroes [8, 11] While the frequency of the other common type of thalassaemia, the $\beta$-thalassaemia has been

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| 5 | 7 | 1 08 | 24 2 |
| 25 | 1 | 0 15 | 3 4 |
| Total | 650 | | |

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which will develop in each case. Thus, newborns with 25% of Hb Barts develop Hb H disease, those with 5% mild microcytic anaemia and those with 1–2% will be normal.

According to the hypothesis of WASI *et al.* [10], Hb H disease occurs from the interaction of two α-thalassaemia genes: one is the classical severe α-thal$_1$, the other is a milder allele, α-thal$_2$. The presence of one α-thal$_1$ gene produces 5% Hb Barts in newborns, whereas the presence of one α-thal$_2$ produces levels of approximately 1–2%. Another interpretation, based on the duplication of the α-chain locus, which also explains the different types of α-thalassaemia, has been proposed by KATTAMIS and LEHMANN [2]. According to this model, the inactivity of one, 2, 3 or of all the 4 α-genes is responsible for the various types of the disease.

Under either hypothesis a family study must be done to assess the incidence of the different thalassaemic genes. Thus, only one parent of patients with Hb H disease (carrying 25% Hb Barts at birth) shows the α-thalassaemia trait, both parents of individuals with 1–2% Hb Barts at birth are normal, whereas two types of inheritance are expected in families of individuals with 5% Hb Barts: either only one parent shows the α-thalassaemia trait, or both are silent carriers. Family studies of the 29 newborns found in this screening gave the following pattern:

*1. Group with 1–2% Hb Barts.* In each family of the 21 cases, only one parent showed mild morphological alterations, the other being completely normal.

*2. Group with 5% Hb Barts.* In this group of 7 newborns, two types of inheritance were found: (a) In two families only one parent was affected by the classical α-thalassaemia trait, with morphological alterations and microcythaemia, while the other was completely normal. (b) In 5 families both parents showed only mild morphological alterations.

*3. Group with 25% Hb Barts.* The only case showing 25% Hb Barts had one parent with alterations typical of the α-thalassaemia trait, the other showing only mild morphological alterations.

Based on these findings, the frequencies of the α-thalassaemia genes were calculated assuming the existence of two allelic genes, α-thal$_1$ and α-thal$_2$ [6]. It turned out that the frequency of the α-thal$_1$ (severe) gene was 0.0023 and that of the α-thal$_2$ (mild) allele was 0.0240. The number of individuals with 1–2, 5 and 25% of Hb Barts found in the study was the expected according to the Hardy-Weinberg law.

The Cuban population derives mainly from two groups: Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids al-

though very small, cannot be neglected, especially in a study on the frequency of α thalassaemia The genetic admixture between these groups has produced an almost continuous variation of the most conspicuous aspects related to the racial pertinency, which makes it very difficult to assess the racial origin However, when a classification based on the colour of the skin thickness of the lips hair, etc is applied to the families reported above, it appears that the three races have introduced the α-thalassaemia genes in Cuba but it is impossible to determine their relative contribution

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*2. Group with 5% Hb Barts.* In this group of 7 newborns, two types of inheritance were found. (a) In two families only one parent was affected by the classical $\alpha$-thalassaemia trait, with morphological alterations and microcythaemia, while the other was completely normal. (b) In 5 families both parents showed only mild morphological alterations.

*3. Group with 25% Hb Barts.* The only case showing 25% Hb Barts had one parent with alterations typical of the $\alpha$-thalassaemia trait, the other showing only mild morphological alterations.

Based on these findings, the frequencies of the $\alpha$-thalassaemia genes were calculated assuming the existence of two allelic genes, $\alpha$-thal$_1$ and $\alpha$-thal$_2$ [6]. It turned out that the frequency of the $\alpha$-thal$_1$ (severe) gene was 0.0023 and that of the $\alpha$-thal$_2$ (mild) allele was 0.0240. The number of individuals with 1–2, 5 and 25% of Hb Barts found in the study was the expected according to the Hardy-Weinberg law.

The Cuban population derives mainly from two groups, Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids, al-

which will develop in each case Thus, newborns with 25% of Hb Barts develop Hb H disease, those with 5% mild microcytic anaemia and those with 1–2% will be normal

According to the hypothesis of WASI *et al* [10], Hb H disease occurs from the interaction of two $\alpha$-thalassaemia genes one is the classical severe $\alpha$-thal$_1$, the other is a milder allele, $\alpha$-thal$_2$ The presence of one $\alpha$-thal$_1$ gene produces 5% Hb Barts in newborns, whereas the presence of one $\alpha$-thal$_2$ produces levels of approximately 1–2% Another interpretation, based on the duplication of the $\alpha$-chain locus, which also explains the different types of $\alpha$-thalassaemia, has been proposed by KATTAMIS and LEHMANN [2] According to this model, the inactivity of one, 2, 3 or of all the 4 $\alpha$-genes is responsible for the various types of the disease

Under either hypothesis a family study must be done to assess the incidence of the different thalassaemic genes Thus, only one parent of patients with Hb H disease (carrying 25% Hb Barts at birth) shows the $\alpha$-thalassaemia trait, both parents of individuals with 1–2% Hb Barts at birth are normal, whereas two types of inheritance are expected in families of individuals with 5% Hb Barts either only one parent shows the $\alpha$-thalassaemia trait, or both are silent carriers Family studies of the 29 newborns found in this screening gave the following pattern

*1 Group with 1–2% Hb Barts* In each family of the 21 cases, only one parent showed mild morphological alterations the other being completely normal

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The Cuban population derives mainly from two groups, Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids, al-

though very small, cannot be neglected especially in a study on the frequency of $\alpha$-thalassaemia The genetic admixture between these groups has produced an almost continuous variation of the most conspicuous aspects related to the racial pertinency, which makes it very difficult to assess the racial origin However, when a classification based on the colour of the skin, thickness of the lips hair, etc is applied to the families reported above, it appears that the three races have introduced the $\alpha$ thalassaemia genes in Cuba, but it is impossible to determine their relative contribution

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# Study of a Large Anglo-Saxon Family with β-Thalassaemia Trait

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*Key Words* Anglo Saxon family Genetics Heterogeneity of β thalassaemia Inheritance of β thalassaemia Thalassaemia

*Abstract* Study of a large Anglo Saxon family with β thalassaemia trait revealed evidence of consanguinity, moreover both branches of the family shared a Spanish ancestor The manifestations of the disorder were varied in severity and yet the degree of severity appeared to breed true within any individual part of the family Our explanation for the inheritance pattern observed in the family was to postulate the existence of two non allelic genes influencing the rate of β chain synthesis

At one time β-thalassaemia was not considered a likely cause of refractory hypochromic anaemia in patients of other than Mediterranean origin Evidence of this disease in Britain is now well documented [1, 2, 5] We have investigated 91 members of a very large apparently Anglo-Saxon family This family is of particular interest for two reasons Firstly, the family is able to trace it s history back five generations to a Spanish sea captain This evidence of Mediterranean ancestry is similar to that found in one British family described by CALLENDER *et al* [1] who had a Spanish ancestor three generations earlier Secondly, despite a consanguinous marriage, there is no evidence of homozygosity, only the heterozygous state, varying in degree of severity, is evident

## *Materials and Methods*

Our proposita was referred to us by her local doctor for confirmation of a suspected β thalassaemia trait As the patient was of Anglo-Saxon origin we decid

*Table I* Haematological results of affected family members shown in figure 1

| Case No | Age | Sex | Hb, g% | MCH, pg | MCV, μm³ | Abnormal red cell morphology | HbA₂ % | HbF, % |
|---|---|---|---|---|---|---|---|---|
| V 1 | 61 | F | 13 9 | 30 1 | 82 | – | 5 0 | 1.2 (1 1) |
| V 2 | 66 | M | 13 1 | NT | NT | + | 8 0 | 6 0 (3 0) |
| VI 1 (proposita) | 37 | F | 11.2 | 21 1 | 61 | + + | 8 3 | 7 1 |
| VI 2 | 34 | F | 13 0 | NT | NT | – | 5 2 | 1 5 (2 0) |
| VI 3 | 35 | F | 10 8 | NT | NT | + + | 7 0 | 6 0 (2 0) |
| VI-4 | 31 | F | 12 5 | 31 8 | 87 | – | 7 5 | 1 1 (0 8) |
| VI-6 | 22 | F | 9 4 | NT | NT | + + | 11 0 | 7 0 (2 0) |
| VII 1 | 12 | M | 10 5 | NT | NT | + + | 8 5 | 3 0 (2 0) |
| VII-6 | 9 | F | 10 3 | NT | NT | + + | 8 0 | 6 0 (3 0) |
| VII 9 | 6 | M | 11 7 | NT | NT | – | 6 0 | 5 0 (3 0) |

HbF control figures for aged blood samples are shown in parentheses

ed to test all the members of the family who could be traced The family proved to be both interested and co-operative The mother of the proposita was able to provide a great deal of detail about the family tree and it transpires that she and her husband are distant cousins having a great grandmother in common

Altogether 91 members representing both branches of the family were investigated Full investigations were not always possible because many members of the family resided in country areas of Australia and the blood samples were often some days in transit Whenever feasible the following investigations were performed by standard procedures full haematological profile from the Coulter Counter Model S, which we have found to be a sensitive index of hypochromia and microcytosis Unfortunately this instrument was not available for testing the initial family members reticulocyte count examination of a stained blood film haemoglobin electrophoresis on cellulose acetate (cellogel) using Tris-EDTA borate barbitone buffer at pH 8 6

$HbA_2$ levels were assessed by dissolving the appropriate bands after staining the strips with Ponceau S and reading the optical density of the resultant solutions in a colorimeter The normal range for this laboratory is 1 7–4 7% with a mean of 3 2%

Fetal haemoglobin (HbF) levels were determined by the alkali denaturation method of BETKE as described by LEHMANN and HUNTSMAN [6] In this laboratory the normal level on fresh blood is <0 8% however in the survey samples were frequently some days in transit and occasionally in poor condition on arrival Whenever possible controls of similar age were used on such occasions and the

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| V 1 | 61 | F | 13 9 | 30 1 | 82 | – | 5 0 | 1 2 (1 1) |
| V 2 | 66 | M | 13 1 | NT | NT | + | 8 0 | 6 0 (3 0) |
| VI 1 (proposita) | 37 | F | 11 2 | 21 1 | 61 | + + | 8 3 | 7 1 |
| VI 2 | 34 | F | 13 0 | NT | NT | – | 5 2 | 1 5 (2.0) |
| VI 3 | 35 | F | 10 8 | NT | NT | + + | 7 0 | 6 0 (2 0) |
| VI-4 | 31 | F | 12.5 | 31 8 | 87 | – | 7 5 | 1 1 (0 8) |
| VI-6 | 22 | F | 9 4 | NT | NT | + + | 11 0 | 7 0 (2 0) |
| VII 1 | 12 | M | 10 5 | NT | NT | + + | 8 5 | 3 0 (2 0) |
| VII-6 | 9 | F | 10 3 | NT | NT | + + | 8 0 | 6 0 (3 0) |
| VII 9 | 6 | M | 11 7 | NT | NT | – | 6 0 | 5 0 (3 0) |

HbF control figures for aged blood samples are shown in parentheses

ed to test all the members of the family who could be traced The family proved to be both interested and co-operative The mother of the proposita was able to provide a great deal of detail about the family tree and it transpires that she and her husband are distant cousins having a great grandmother in common

Altogether 91 members representing both branches of the family were investigated Full investigations were not always possible because many members of the family resided in country areas of Australia and the blood samples were often some days in transit Whenever feasible the following investigations were performed by standard procedures full haematological profile from the Coulter Counter Model S which we have found to be a sensitive index of hypochromia and microcytosis Unfortunately this instrument was not available for testing the initial family members reticulocyte count examination of a stained blood film haemoglobin electrophoresis on cellulose acetate (cellogel) using Tris EDTA borate/barbitone buffer at pH 8 6

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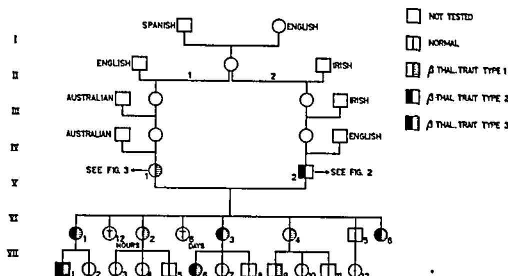



*Fig 1* Abbreviated pedigree of the proposita (VI-1) illustrating the consanguinity and the Spanish ancestry

*Table II* Haematological results on affected family members shown in figure 2 (except V 2)

| Case No | Age | Sex | Hb, g% | MCH, pg | MCV, µm³ | Abnormal red cell morphology | HbA₂, % | HbF, % |
|---|---|---|---|---|---|---|---|---|
| V-7 | 59 | M | 10 8 | 21 6 | 69 | + + | 5 5 | 1 0 (0 8) |
| VI-13 | 22 | F | 12 5 | 30 7 | 84 | – | 5 7 | 1 1 (0 8) |
| VI-17 | adult | F | 11 1 | 20 7 | 66 | + | 8 8 | 3 0 (1 0) |
| VII-16 | 34 | M | 11 7 | 21 3 | 64 | + + | 8 4 | 2 7 (1 0) |
| VII-18 | 25 | F | 12 9 | 29 7 | 86 | – | 6 5 | 0 8 |
| VII-25 | 9 | F | 12 2 | 20 0 | 57 | + + | 5 0 | 2 8 (1 1) |
| VIII-1 | 6 | F | 9 8 | 20 3 | 61 | + + | 8 1 | 3 0 (1 0) |
| VIII-2 | 9 | F | 10 7 | 20 2 | 61 | + + | 7 5 | 3 1 (1 0) |
| VIII-3 | 2 | M | 10 3 | 19 8 | 60 | + + | 10 4 | 3 1 (1 0) |

VI 8, VI-18 tested at another hospital and diagnosed HbF control figures for aged blood samples are shown in parentheses

results interpreted accordingly In tables I, II, the appropriate control figure for any aged sample is shown in parentheses

Serum iron levels were not determined because many of the family were from country areas and suitable sera were difficult to obtain Moreover some were currently receiving iron therapy

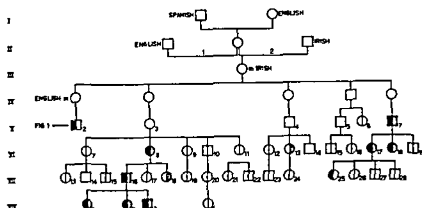



*Fig 2* Paternal branch of the family For explanation of symbols, see fig 1

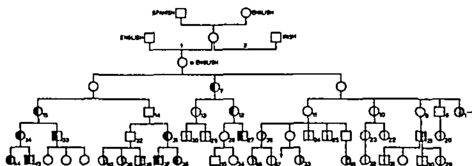



*Fig 3* Maternal branch of the family For explanation of symbols, see fig 1

## *Results*

For convenience, the family tree has been greatly simplified and divided into three sections Figure 1 shows the pedigree of the proposita and her immediate family, figure 2 depicts her father's family and figure 3 the maternal branch of the family The haematological results are similarly divided and summarised in tables I–III, except for VI–8 and VI–18 who were investigated and diagnosed at other hospitals

Thirty-nine family members of the 91 investigated showed evidence of β-thalassaemia trait in some form Thirteen members (from both branches) showed an elevated $HbA_2$ level with no other detectable ab-

*Table III* Haematological results of affected family members shown in figure 3 (excep V-1)

| Case No | Age | Sex | Hb, g% | MCH, pg | MCV, $\mu m^3$ | Abnormal red cell morphology | $HbA_2$, % | HbF, % |
|---|---|---|---|---|---|---|---|---|
| IV-7 | >80 | F | 12 0 | 21 4 | 67 | + | 7 1 | 2 4 (1 0) |
| V-12 | adult | F | 11 9 | 22 8 | 74 | + | 5 9 | 3 0 (0 8) |
| V-15 | adult | F | 11 0 | 21 3 | 66 | + | 8 4 | 4 4 |
| VI-21 | 54 | M | 16 5 | 32 4 | 96 | – | 6 6 | 0 9 |
| VI 27 | child | M | 12 7 | 19 8 | 64 | + | 6 2 | 1 0 (0 8) |
| *VI 31* | *40* | *F* | *11 6* | *19 9* | *58* | + | *10 3* | *1 2 (0 8)* |
| VI-33 | 37 | M | 13 2 | 22 7 | 75 | + | 6 7 | 2 0 (0 8) |
| VI-34 | adult | F | 10 2 | 21 0 | 63 | + + | 7 5 | 1 7 (1 0) |
| VI 35 | 49 | F | 14 1 | 31 5 | 89 | – | 7 0 | 1 0 (0 8) |
| VII-33 | adult | F | 13 4 | 31 5 | 90 | – | 6 0 | 0 9 |
| VII-36 | child | F | 11 5 | NT | NT | + | 8 0 | 3 7 |
| *VII 37* | *13* | *M* | *11 5* | *19 3* | *56* | + | *10 8* | *1 7 (0 8)* |
| VII-38 | child | F | 12 9 | 29 9 | 82 | – | 6 3 | 0 9 |
| VII 39 | child | M | 12 7 | 29 7 | 82 | – | 5 6 | 0 7 |
| VII-40 | child | F | 13 1 | 29 3 | 82 | – | 5 3 | 1 0 |
| VII-43 | 6 | M | 10 5 | 19 0 | 57 | + + | 8 6 | 1 7 (1 0) |
| VII-44 | 4 | F | 11 2 | 20 2 | 60 | + + | 8 8 | 3 0 (1 0) |
| VII-45 | 23 | F | 13 0 | NT | NT | – | 6 4 | 0 8 |
| VII 46 | 23 | F | 12 7 | 28 3 | 79 | – | 5 4 | 1 4 (1 0) |

HbF control figures for aged blood samples are shown in parentheses

normality Of the remaining $\beta$-thalassaemia traits in the family, all had elevations of both $HbA_2$ and *HbF levels* and *15* were also *anaemic In* the case of V–7, the anaemia and marked red cell changes may have been accentuated by the co-existence of multiple myeloma

## *Discussion*

It is now generally accepted that the abnormality in the thalassaemias lies not in the structure of the globin chains but in the quantity of the particular chain produced The actual mechanism whereby chain production is decreased is as yet unknown Recently, it has been shown [3

*Table IV* Haematological results of family Z. and family S

| Case No | Age | Sex | Hb, g % | MCH, pg | MCV, μm³ | Abnormal red cell morphology | HbA₂, % | HbF, % |
|---|---|---|---|---|---|---|---|---|
| *Family Z* | | | | | | | | |
| Y Z, proposita | 40 | F | 8 9 | 18 7 | 67 | + + + | 10 | 6 5 |
| Husband R Z. | 43 | M | 14 6 | 29 3 | 86 | – | 3 0 | 0 5 |
| Brother J M | 50 | M | 14 5 | NT | NT | – | 1 7 | 1 0 |
| Sister N B | | F | 12 2 | 25 2 | 72 | – | 5 4 | 1 0 |
| Daughter T Z | 21 | F | 12.6 | 23 1 | 67 | + | 6 6 | 0 6 |
| Daughter C. Z.[1] | 18 | F | 12.2 | 21 9 | 67 | + | 4 8 | 0 4 |
| Daughter S Z.[1] | 18 | F | 12.3 | 23 7 | 70 | + | 5 5 | 0 8 |
| *Family S* | | | | | | | | |
| C S, proposita | 30 | F | 8 3 | 19 3 | 59 | + + + | 8 4 | 1 8 |
| Husband V S | 30 | M | 14 1 | NT | NT | – | 2 5 | 0 8 |
| Daughter K S | 6 | F | 14 1 | 24 4 | 70 | + | 6 7 | 1 1 |
| Son J S | 2 | M | 11 5 | 23 0 | 66 | + | 10 | 1 7 |

[1] Non identical twins

4] that the actual amount of βmRNA in β-thalassaemia is lower than normal This could result from a β structural gene altered to produce βmRNA less efficiently, or a separate thalassaemia' gene exerting an inhibitory effect on the β-structural gene

β-Thalassaemia trait manifests with varying degrees of severity, however, it is usually fairly constant in its manifestations in any one family It is apparent from the results that these degrees of severity exist *throughout both sides of this family*

To facilitate the discussion it has been necessary to allot an arbitrary classification for the patterns of β thalassaemia trait seen in this family For convenience we designated them as follows Type 1 normal with the exception of an increase in HbA₂, type 2 deficient haemoglobinisation and abnormal red cell morphology, increased levels of HbA₂ and HbF, type 3 symptomatic anaemia, deficient haemoglobinisation with more marked changes in red cell morphology, increased levels of HbA₂ and HbF It may appear that by attempting such a classification we are drawing too fine a line of distinction between the variations of β thalassaemia trait We must stress therefore that the blood films were exam-

# Heterozygous and Homozygous Factor XI Defect in a Consanguineous Family

## Effect of Age on Heterozygous Expression

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*Key Words* Age effect on factor XI Bleeding disorders Coagulation Factor XI defect

*Abstract* A woman with symptoms of prolonged post traumatic bleeding was found to have a severe defect of factor XI, and was thought to be homozygously af fected Both her parents who were first cousins, and all her three children though asymptomatic, showed a moderate defect and appeared to be heterozygous Both her parents factor XI levels were about twice those in her children and this was thought to be an age effect

Various clotting factors may rise or fall with age [1–3], factor XI is reported [1] to rise with age in women but to decrease in men We report a consanguineous Jewish family transmitting a factor XI defect, the pro band appears to be homozygously affected and heterozygotes can be identified in both the previous and subsequent generations The older heterozygotes, a man and a woman, have much higher factor XI levels than the three younger heterozygotes suggesting that the levels in both the grand parents have risen with age

### *Laboratory Methods*

*Blood samples* were anticoagulated with trisodium citrate dihydrate solution For the preliminary tests 9 vol of blood were mixed with 1 vol 3.2% solution and for the factor XI assays 49 vol of blood were mixed with 1 vol 20% solution Samples were handled and tested throughout in plastic equipment

*The one stage prothrombin time test* was performed with Manchester Comparative Reagent (phenolized suspension of human brain) and 25 mM calcium chloride

solution, and the result expressed as the ratio of the patient's time to the control time

*The partial thromboplastin time with kaolin* involved an initial preincubation of equal volumes of the patient's citrated plasma and a suspension of light kaolin 5 mg/ml in Tris buffer 50 mM (pH 7.4 at room temperature) for 10 min, the reaction mixture was then completed by adding volumes of Thrombofax (Ortho) and 25 mM calcium-chloride solution. The upper limit of normal is taken as 6 sec longer than the control [4]

*Contact product* was prepared by PÉREZ REQUEJO's modification [5] of NOSSEL's method [6] As a screening test for contact factor defects, the partial thromboplastin time test was repeated, adding a volume of contact product after the calcium-chloride solution instead of the initial preincubation with kaolin If a prolonged result with kaolin was normalised by contact product, the plasma sample was considered to be defective in contact factor activity [7]

*In factor XI assays* for family members III3, III6, IV1, IV2, V1, V2 and V3 (fig 1) natural factor XI deficient plasma was available as substrate For members IV4, V4, V5 and V6, an artificial factor XI deficient substrate [8] was used The assays were performed as partial thromboplastin times with kaolin on aliquots of the substrates to which had been added three serial dilutions of the test plasma or, as a standard, three dilutions of a pool of three fresh normal plasmas. The factor XI activities were calculated from the results by the standard methods for parallel line bioassays [9]

All tests were performed at 37 °C.

## *Case Report and Family Details*

The family tree is shown in figure 1 No details are available for generations I and II In generation III, members 3 and 6 are the grandparents of the present study Neither has unequivocal symptoms of abnormal bleeding although III6 bled 'severely' after an operation for prolapse, but the bleeding was controlled by packing and she was not transfused

The proband, IV2, bled for 'some days' and for 2 days after two dental extractions She bled in the night following an operation for prolapse and she was transfused 3.5 litres blood A haematoma formed after a breast operation and the wound bled intermittently for 1 month She has had menorrhagia only since the birth of her children Results of her preliminary clotting tests are given in table I Her husband, IV1, who is her second cousin, has no history of abnormal bleeding Her sister, IV4, suffered a post partum haemorrhage after the birth of her first child for which she received a transfusion of plasma but whole blood was not given, otherwise, she has had 'numerous' dental extractions with a normal haemostatic response None of the six children in generation V have any history of defective haemostasis, V5 has had teeth extracted and V6 tonsillectomy without abnormal bleeding

Factor XI levels, ABO blood groups and dates of birth are given in figure 1 The figure also shows that III3, IV2 and V1 suffer from Raynaud's phenomenon, proba

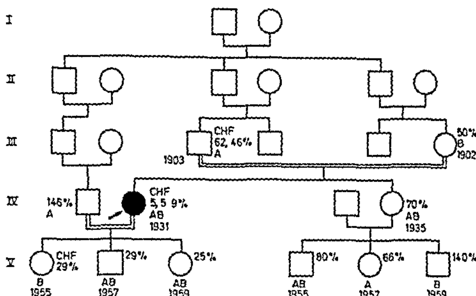



*Fig 1* Family tree Factor XI%, A and B blood groups, year of birth and the incidence of 'cold hands and feet' (CHF Raynaud's phenomenon) are shown Arrow indicates proband who had symptoms of abnormal bleeding

*Table I* Preliminary clotting tests in proband

| Test | Result | |
|---|---|---|
| Prothrombin time ratio | 0 93 | |
| Partial thromboplastin time with kaolin, sec | | |
| Control | 36 9 | |
| Patient | 48 4 | |
| Difference from control | | +11 5 |
| Control + patient 50 50 | 37.2 | |
| Difference from control | | +0 3 |
| Partial thromboplastin time with contact product, sec | | |
| Control | 40 1 | |
| Patient | 38 4 | |
| Difference from control | | −1 7 |

The patient's partial thromboplastin time with kaolin was prolonged, but was corrected by equal mixture with normal plasma, substituting contact product for kaolin activation also corrected the patient's abnormality

bly all since childhood, fingers and toes blanch in the cold but there have been no complications

*Operative cover in the proband* The proband subsequently underwent an anterior and posterior perineal repair Preoperative cover was provided by approximately 1 litre of fresh noncontacted plasma, and her factor XI level rose from 6 to 37%. Similar doses of frozen uncontacted plasma were given after 11 and 23 h, after the second her level rose from 29 to 36%. Tranexamic acid, 1 g every 6 h was also given by mouth from the preoperative day for 7 days, and for a further week at 0.5 g every 6 h, in addition, a supplementary intravenous dose of 3 g ε aminocaproic acid was given preoperatively Bleeding at operation was thought to be somewhat excessive but haemostasis was secured postoperative bleeding was not abnormal

## Discussion

The distribution of A and B blood groups confirm the family relationships shown The simplest interpretation of the distribution of factor XI levels and the occurrence of bleeding symptoms is that proband IV2 is homozygous for the defective gene, and that both her parents and all her children are heterozygotes It is then necessary to explain why her parents' levels of factor XI are approximately twice the levels in her children, and it seems likely that this is an age effect Her parents are some 55 years older than her children, and an average increase of approximately 0.5% per annum for both the grandparents would account for the difference, a value within the range for factors VII and IX found by others [2, 3] This is contrary to the evidence of HAMILTON *et al* [1] who reported a rise in females but a fall in males with age among normal subjects

The haemostatic responses of the heterozygotes, and of the proband at her various operations, suggest that at least for this particular mutation, 25–30% of factor XI activity seems to be adequate for normal haemostasis, though 5–10% does not.

*Acknowledgements* We are grateful to Dr J EASTON for referring the proband to us, and to Miss M S CORK and Miss J PHILLIPS for carrying out the factor XI assays We are grateful to Dr J H RENWICK who kindly discussed the genetics of this family with us

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# Circulating IgG Antibodies against Factors IX and VIII in Multiple Sclerosis[1]

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*Key Words* Antibodies against clotting factors Anticoagulants Blood coagulation Factor VIII inhibitor Factor IX inhibitor Multiple sclerosis

*Abstract* A 64-year-old man with multiple sclerosis developed a circulating anticoagulant Three immunoglobulin fractions (I = IgG II = IgG+IgA III = IgM) were separated from patient serum by 33-percent ammonium sulfate precipitation and DEAE 52 cellulose column chromatography Fractions I (IgG) and II (IgG+IgA) had factor VIII inhibitory activity on incubation Fraction I (IgG) possessed in addition immediate anti IX activity which did not require incubation Absorption studies characterized the VIII inhibitors as IgG $\varkappa$ in fraction I and IgG $\varkappa$+IgG $\lambda$ in fraction II Immediately reactive IgG anti IX antibodies associated with kinetically distinct IgG anti VIII antibodies have not been described previously

Circulating inhibitors of factor VIII [1] have appeared in previously transfused hemophilia A patients [2], in normal women following parturition, in elderly individuals, and in patients with immunological disorders [3] These inhibitors often have been IgG antibodies [1] although IgA and IgM VIII inhibitors have been described [4, 5] The IgG antibodies frequently have reacted predominantly with anti-$\varkappa$ light-chain antisera [1 6] and in several patients have been subclassified as IgG-4 or IgG 3 [7, 8] Incubation of normal plasma with VIII inhibitors often has resulted in further loss of VIII activity [1, 6, 9] Most acquired inhibitors of factor IX have been detected in previously transfused hemophilia B patients [1] These IgG antibodies (IgG-4 $\lambda$ in 1 patient [10]) have produced immediate

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interference with the IX activity of normal plasma without further decrease after incubation [1, 11]

We have studied a nonhemophilic patient with IgG antibodies inhibiting the activities of both factor IX and factor VIII

## Case Report

Patient S S, a 64 year-old white male, developed diplopia and ataxia He was found to have generalized hyperreflexia with clonus bilateral Babinski and Hoffman signs, bilateral optic pallor, poor memory and calculating ability, and labile affect There were diffuse electroencephalographic abnormalities Skull films and brain scan were normal γ globulins were increased in a polyclonal electrophoretic pattern in serum and cerebrospinal fluid Additional studies included normal hemoglobin white cell count and differential platelet count, prothrombin time (PT) and activated partial thromboplastin time (APTT) Erythrocyte sedimentation rate was 115 mm/h Antinuclear antibodies and rheumatoid factor were not present. Serum chemical profile was normal, as were serum $B_{12}$ and folate levels

Three years later, he developed hematuria and extensive ecchymoses There was no previous bleeding history and no family history of hemorrhagic disorder He had never received a blood transfusion On admission the hemoglobin was 9.2 g%, reticulocytes 0 8%, white cell count 6 200 (normal differential) and platelets 382,000/μl Red cells were microcytic and hypochromic. Table I summarizes initial coagulation studies Additionally, the following were normal Ivy bleeding time (3 min, 15 sec), tourniquet test clot retraction, fibrin degradation products (<10 μg/ml) thrombin time (patient 20 sec/control 20 sec), and fibrinogen (202 mg%) Hemolytic assays of serum complement components [12] were normal serum $CH_{50}$ values were 256 000 units/ml for C4 and 19,200 units/ml for C3

## Materials and Methods

Plasma recalcification time (PRT) assays [13] were performed by adding 0 2 ml of 0 02 M $CaCl_2$ to 0 2 ml plasma For crossed PRT determinations patient and control plasmas were mixed to 0 2 ml final volumes and tested immediately Inhibitor concentration was estimated by diluting patient plasma in Tris buffered saline (Tris HCl 0 05 M+NaCl 0 15 M pH 7 4) to a final volume of 0.2 ml prior to the addition of 0 2 ml fresh normal plasma and 0 2 ml 0 02 M $CaCl_2$

APTT and PT determinations were done using standard techniques

Assays for coagulation factors [13] were done using single factor-deficient plasmas obtained from Dade Reagents Factors XII XI IX, and VIII were assayed by methods based on the one stage APTT and assays for factors VII, V and prothrombin were done by a one stage PT method Factor X was assayed by both APTT and PT methods

Separation and purification of serum immunoglobulins were done by 33 per cent ammonium sulfate precipitation [14] and DEAE 52-cellulose (Reeve Angle

Clifton, N J ) anion exchange chromatography [15] using a continuous gradient $KH_2PO_4/K_2HPO_4$ (pH 8 0) buffer Column fractions I, II and III were dialyzed against Tris NaCl and adjusted to concentrations of 7 mg/ml Immunoglobulins were identified by immunodiffusion [16] against antibodies to IgG, IgA, IgM IgD, IgE, $\varkappa$, and $\lambda$ light-chains (Meloy Laboratories, Springfield, Va.) Anticoagulant activity in fractions I, II, and III was determined by mixing 0 05 ml with 0 05 ml of fresh normal plasma and performing APTT determinations and specific factor assays immediately and following incubation for 1 h at 37 °C.

Antisera to IgG IgA, IgM, $\varkappa$, and $\lambda$ light-chains were absorbed for 1 h at 23 °C with 100 mg/ml $BaSO_4$ centrifuged, and incubated for 30 min at 56 °C to destroy coagulation factor activity in the antisera [10, 11] The column fractions were combined with absorbed antisera in ratios ranging from *1 1 to 1 8 (fraction antisera)* and incubated at 37 °C for 1 h and at 4 °C for 16 h. Each antiserum treated column fraction (0 1 ml total volume) was added to 0 1 ml fresh normal plasma, and factor assays were performed immediately and following incubation at 37 °C for 1 h Control factor levels were determined by (1) combining 0 1 ml fresh normal plasma with 0 1 ml Tris NaCl (to determine dilutional effect on factor levels), and (2) incubating each column fraction with Tris NaCl at 37 and 4 °C in ratios equivalent to those with antisera prior to performing factor assays (to determine inhibitory activity in diluted fractions) Each column fraction antiserum combination was tested in immunodiffusion plates versus pooled normal human serum Precipitin lines indicated antiserum in excess of column fraction immunoglobulins

## *Results*

*Initial coagulation studies* Patient S S had an inhibitor directed against the intrinsic coagulation pathway (table I) Factor X activity in patient plasma was undetectable by APTT method and 80% by PT assay Patient plasma required a 50- to 100-fold dilution to eliminate the anticoagulant effect.

*Characterization of anticoagulant* Three serum fractions containing immunoglobulins were obtained by DEAE-52 cellulose chromatography IgG (fractions I and II), IgA (fraction II), and IgM (fraction III) IgD and IgE were not detected Only fractions I and II had anticoagulant activity The mixture of fraction I with fresh normal plasma resulted in an immediate 50-percent decrease in factor IX activity not accentuated by incubation In contrast, anti-VIII activity was not detected immediately, however, after incubation of fraction I or fraction II with fresh normal plasma VIII activity was reduced by >90% There were no inhibitory effects on the activities of factors XII, XI, or X by either fraction

The anti VIII effect of fraction I was 80% reversed by absorption with IgG antiserum and completely reversed by absorption with $\varkappa$-anti

*Table I* Initial coagulation studies

| | Plasma recalcification time (seconds) | Activated partial thromboplastin time (seconds) | Prothrombin time (seconds) |
|---|---|---|---|
| Patient plasma | >400 | 97 | 11 |
| Normal plasma | 100 | 35 | 11 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Patient plasma | 100% | 75% | 50% | 25% | 10% | 0 |
| Normal plasma | 0 | 25% | 50% | 75% | 90% | 100% |
| Plasma recalcification time (seconds)[1] | >400 | 253 | 218 | 202 | 195 | 100 |

| | Activated partial thromboplastin time method[2] | | | | | Prothrombin Time Method[3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Factor* | *XII* | *XI* | *VIII* | *IX* | *X* | *X* | *VII* | *V* | *II* |
| Patient plasma activity (%) | 1 7 | <5 | 3 1 | 3 5 | 0 | 80 | 85 | 88 | 103 |

[1] Final volume 0 2 ml
[2] Patient plasma 0 1 ml + ellagic acid phospholipid 0 1 ml + 0 02 M $CaCl_2$ 0 1 ml
[3] Patient plasma 0 1 ml + thromboplastin − $CaCl_2$ 0 2 ml

serum Partial reversal of fraction II VIII-inhibitory activity followed absorption with IgG (40%), $\kappa$ (60%), and $\lambda$ (55%) antisera Ratios of fraction to antisera were 1 8 At this dilution antisera to $\kappa$- and $\lambda$-light-chains were in excess with fractions I and II, IgA antiserum was in excess with fraction II, and IgG antiserum was in excess with fraction II Anti-VIII activity was not consistently present at fraction I dilutions exceeding 1 8 It was not possible to characterize the immediate anti-IX activity in fraction I since dilutions in excess of threefold did not possess effects which could be reversed by fraction I antisera ratios less than 1 3

## *Discussion*

Patient S S developed a severe hemorrhagic disorder associated with multiple sclerosis An anticoagulant with immediate inhibitory activity

against the intrinsic coagulation pathway was detected by initial studies (prolonged PRT and APTT, normal PT, abnormal crossed PRT), and very low levels of factors XII, XI, VIII, IX, and X were found However, normal levels of X, as well as VII, V, and prothrombin, were obtained in assays using a one stage PT method The normal factor X by PT assay and undetectable X activity by APTT assay suggested that the anticoagulant was directed against the intrinsic activator of factor X, either activated factor IX (IXα) or the complex of phospholipid, $Ca^{++}$, factor VIII, and IXα [17]

Anion exchange chromatography separated two serum immunoglobulin fractions with anticoagulant properties Fraction I (IgG) had immediate inhibitory effects on factor IX activity, and fractions I (IgG) and II (IgG+IgA) inhibited factor VIII activity following incubation The IgG VIII inhibitor in fraction I was exclusively of the κ light-chain type Because the anti IX effect of fraction I IgG disappeared upon 1 3 dilution it was not possible to determine with antiserum absorption experiments whether anti IX antiobdy was monoclonal or polyclonal IgG antibodies of both κ- and λ light-chain types were responsible for the anti-VIII effects of fraction II Fraction II IgG antibodies may have been predominantly of IgG-4 subclass, since IgG-4 (which is more positively charged than IgG 1, IgG 2, and IgG 3 [18, 19]) is eluted with IgA from anion exchange columns Acquired VIII inhibitors in other patients have been characterized as IgG-4 antibodies [7, 8]

IgG-4 does not activate complement via the C1 pathway, and IgG-2 does so poorly when compared with IgG 1 and IgG 3 [20] The normal serum C4 and C3 levels implied that excessive complement activation through the C1 or C3 bypass (properdin) pathways [21] did not result from IgG reactions with factor IX or factor VIII Thus, the IgG VIII inhibitor in fraction II may have been IgG-4, and the anti IX and anti VIII antibodies in fraction I may have been IgG 2

Factor VIII and IX assays by one stage APTT methods involve the generation of phospholipid, $Ca^{++}$, VIII IXα complexes following the addition of test plasma dilutions to VIII- or IX-deficient plasma If the fraction I IgG had been directed against the VIII IXα complex than immediate inhibition of normal plasma VIII activity and further decrease in IX activity with incubation should have been found However, the kinetic behavior of the fraction I IgG inhibitors of IX and VIII were distinct Apparently different IgG antibodies in fraction I inhibited the activities of factors IX and VIII

It is unlikely that the IgG antibodies reacted with phospholipid components of the complex involving VIII, IXa, and Ca⁺⁺, since neither fraction I nor fraction II inhibited X activity (which requires formation of Xa V-phospholipid Ca⁺⁺ complexes) Phospholipid may be the plasma component against which the lupus anticoagulant [1] is directed Immediately reactive IgG and/or IgM antibodies [22 23] interfere with coagulation in some patients with systemic lupus erythematosus and other immunological disorders [1] The lupus anticoagulant is usually associated with prolonged PT, and bleeding has infrequently occurred in the absence of thrombocytopenia or reduced prothrombin levels [1]

Anticoagulants against factor IX occur following transfusion in some patients with Christmas disease Acquired factor IX deficiency has been reported in systemic lupus erythematosus [24] rheumatic fever [25] and myeloid metaplasia [25] Patient S S is unique in that both anti IX and anti-VIII IgG antibodies were present in association with multiple sclerosis

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# Infectious Mononucleosis in a Patient with *Hodgkin's Disease*

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*Key Words* Epstein Barr virus  Hodgkin's disease  Infectious mononucleosis

*Abstract* The case is presented of a patient, treated for Hodgkin's disease, who contracted infectious mononucleosis more than 3 years later While Epstein Barr virus has been considered a possible etiologic factor in Hodgkin's disease, the sequence of events reported in this case has to be interpreted as evidence against a causal relationship between the virus and Hodgkin's disease in this patient

The prior occurrence of infectious mononucleosis reported in several cases of Hodgkin's disease [8, 18] might point to a relationship between both diseases In an extensive investigation, 29 cases have been found, with an interval from the diagnosis of infectious mononucleosis to that of Hodgkin's disease of less than 1 year in eight [8] In three cases the infectious mononucleosis related lymphadenopathy persisted and merged into the subsequent Hodgkin's disease In a cohort study of cancer following infectious mononucleosis in Connecticut [2], reports of 4,529 cases of infectious mononucleosis could be identified through existing records and matched against a register of cancer cases because both are reportable diseases in that state Relative risks were found elevated for certain forms of cancer, the largest risk being that for malignant lymphoma (7 cases observed, 1 9 expected, relative risk 3 7) In five cases infectious mononucleosis preceded Hodgkin's disease by 3–11 years In another cohort study [15] no significant differences in mortality from Hodgkin's disease were found in a group of 2,437 veterans with infectious mononucleosis diagnosed in 1944 and a similar number of controls Sternberg-Reed cells, until recently considered pathognomonic for Hodgkin's disease, have been observed in lymph

nodes of patients with infectious mononucleosis [12] and in recurrent Burkitt lymphoma [19]

There is widespread agreement now that infectious mononucleosis is brought about by Epstein-Barr virus (EBV) in individuals who were seronegative for EBV before [reviews 9, 14] Although a strong serologic and virologic relationship exists between the virus and Burkitt lymphoma and nasopharyngeal carcinoma [9, 14] an etiologic role for EBV in either malignancy cannot be considered established Several investigators have found a serologic relationship between EBV and Hodgkin's disease Both anti-EBV virus capsid antigen (VCA) antibodies, determined by an indirect immunofluorescence technique on fixed EBV-carrying lymphoblastoid cell lines [5, 7, 10, 11], and antibodies, reacting with specific cell surface antigens [7] were significantly elevated in patients as opposed to controls No difference in titers of antibody to the closely related cytomegalovirus (CMV) could be demonstrated between patient and control groups [11] These antibodies were assayed by the relatively insensitive complement fixation technique We therefore measured antibody titers to both EBV-VCA and CMV in sera of untreated patients with Hodgkin's disease and in age- and sex matched controls, using the sensitive indirect immunofluorescence technique [10] Both antibody levels were found to be significantly elevated in patients as opposed to controls These results cast some doubt on a specific role of EBV in Hodgkin's disease The finding of EBV-seronegative patients [10, 11] may be interpreted as evidence against an etiologic role for EBV at least in these cases of Hodgkin's disease

The occurrence of infectious mononucleosis in a patient, treated more than 3 years before with curative radiotherapy for Hodgkin's disease, is reported in this paper It is considered clear evidence of a lack of causal relationship between EBV and Hodgkin's disease in this patient

## *Methods*

Antibody titers against EBV-VCA and EBV-associated nuclear antigen (EBNA) were titrated in pre-illness, acute phase and reconvalescent sera of the patient.

The indirect immunofluorescence technique for the detection of anti-EBV VCA antibodies was performed as described before [10]. The indirect immunofluorescence test for anti EBNA antibodies was a modification of procedures described before [4, 17]. The substrate was the P3HR1 cell line The cells were brought onto slides, air-dried and fixed in acetone at − 20°C for 10 min Patient sera were inactivated at 56°C for 30 min and applied to the cells in twofold dilutions. The preparations were then incubated for 30 min at room temperature, washed and incubated for 30 min at room temperature with

a complement-containing serum without detectable antibodies to EBV, diluted 1 6. After further washings FITC-conjugated anti human $\beta$1C/$\beta$1A globulin (Hyland Laboratories), diluted 1 30 was applied After incubation and washings, the slides were dried and mounted with coverslips and glycerol phosphate-buffered saline (PBS) Dilutions were made in PBS Known positive and negative sera were included Human diploid embryonic fibroblasts were used as negative substrate. A positive reaction was characterized by a finely granular staining of the nuclei of almost all P3HR1 cells

## Case Report

The patient was a 17 year-old boy at his first admission in 1971 He then underwent diagnostic thoracotomy for a large right sided mediastinal mass Pathologic examination revealed the nodular sclerosis type of Hodgkin s disease. He was staged CSIAPSIs-n + u [1] Examination of peripheral blood leukocyte concentrates and spleen histology led to the assignment into the localized form [3] Head and neck, mediastinal and axillary glands were irradiated in a single so-called mantle field (Cobald, 4 000 rad in 38 days) He remained healthy until November 1974, more than 3 years after the irradiation He was readmitted with a history of fever up to 39°C, headache and sore throat of 12 days duration Physical examination revealed a generalized lymphadenopathy Hemoglobin 15 9 g/100 ml, hematocrit 47%, white blood cell count 28 800/µl with 79% mononuclear cells among which were 71% atypical lymphocytes, platelet count 271,000/µl Serum alkaline phosphatase 30 Bessey units (normal 0 8–3 5), SGOT 143 Wroblewski units (normal 5–40), SGPT 194 Wroblewski units (normal 5–35), bilirubin <1 mg/100 ml Monosticon test positive, heterophil titer ≥ 1 512 after guinea pig kidney absorption Anti EBV antibody titers are summarized in table I A seroconversion for both anti EBV VCA and anti EBNA antibodies could be demonstrated in the acute phase serum as opposed to pre-illness sera The diagnosis was infectious mononucleosis All symptoms and signs faded off in a few weeks The patient is healthy and without signs of Hodgkin's disease at the time of writing (February 1975)

## Discussion

The diagnosis of infectious mononucleosis in this patient is justified by the clinical and laboratory findings, including a high heterophil titer after guinea pig kidney absorption It is confirmed by the seroconversion concerning anti-EBV VCA and anti-EBNA antibodies Obviously, this patient contracted a primary infection with EBV more than 3 years after curative treatment of Hodgkin's disease Therefore EBV cannot be considered an etiological factor in the origin of Hodgkin's disease in this patient It is tempting to speculate on an etiologic role for herpesviruses in lymphoma At least four herpesviruses have been held responsible for lymphoma in animals Marek's virus in chickens [16], *Herpesvirus saimiri* and *Herpesvirus*

*Table I* Serological data before, during and after infectious mononucleosis in a patient with Hodgkin's disease

| *Days before (−) and after (+) onset of symptoms* | *Anti EBV antibody titers* | | *Heterophil titer after guinea pig kidney absorption* |
|---|---|---|---|
| | anti EBV VCA | anti EBNA | |
| − 1138 | neg. | | |
| − 955 | neg. | neg. | |
| + *15* | *1,280* | *4* | *> 512* |
| + 79 | 1,280 | 2 | |

neg = Anti EBV VCA titer < 10, anti EBNA titer < 2.

*ateles* in monkeys [13] and *Herpesvirus sylvilagus* in rabbits [6] As Hodgkin's disease may well represent an etiologically heterogeneous group, it cannot be excluded that EBV could trigger the disease process in individual cases

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# Types of Leukemia in Chronic Benzene Poisoning. A Study in Thirty-Four Patients[1]

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*Key Words* Benzene poisoning Leukemia Preleukemia Shoe workers

*Abstract* The distribution of various types of leukemia due to chronic exposure to benzene is described in a series comprising 34 cases The incidence of leukemia among 31 shoe workers was 13.5/100 000 Acute myeloblastic leukemia was the most frequent type followed by preleukemia, acute erythroleukemia and acute lymphoblastic leukemia. The extreme rarity of chronic myeloid leukemia was a noteworthy finding The differences and similarities between the distribution of various types of leukemia in different series of patients with chronic exposure to benzene and ionizing radiation are discussed.

The etiology of leukemia is still obscure Despite this fact, the role of ionizing radiation and benzene in the development of leukemia is well-known [3–5, 8, 9, 11–17, 19] During the period from 1967 to 1973, we collected 26 shoe-workers chronically exposed to benzene with acute leukemia in Istanbul and showed that the incidence of leukemia among them was 13/100,000 which is a marked and statistically significant increase over that of leukemia in the general population [3] In addition, in 1974 we have observed 8 new individuals with leukemia associated with chronic benzene poisoning The purpose of this paper is to report them and compare the types of leukemia in individuals chronically exposed to benzene and those exposed to ionizing radiations

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# Types of Leukemia in Chronic Benzene Poisoning. A Study in Thirty-Four Patients[1]

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*Key Words* Benzene poisoning Leukemia Preleukemia Shoe workers

*Abstract* The distribution of various types of leukemia due to chronic exposure to benzene is described in a series comprising 34 cases The incidence of leukemia among 31 shoe workers was 13.5/100,000 Acute myeloblastic leukemia was the most frequent type followed by preleukemia, acute erythroleukemia and acute lymphoblastic leukemia The extreme rarity of chronic myeloid leukemia was a noteworthy finding The differences and similarities between the distribution of various types of leukemia in different series of patients with chronic exposure to benzene and ionizing radiation are discussed.

The etiology of leukemia is still obscure Despite this fact, the role of ionizing radiation and benzene in the development of leukemia is well-known [3–5, 8, 9, 11–17, 19] During the period from 1967 to 1973, we collected 26 shoe-workers chronically exposed to benzene with acute leukemia in Istanbul and showed that the incidence of leukemia among them was 13/100,000 which is a marked and statistically significant increase over that of leukemia in the general population [3] In addition, in 1974 we have observed 8 new individuals with leukemia associated with chronic benzene poisoning The purpose of this paper is to report them and compare the types of leukemia in individuals chronically exposed to benzene and those exposed to ionizing radiations

[1] This study was supported by a grant (TAG/328) from the Scientific and Technical Research Council of Turkey

## Material and Methods

According to the official records there are 28,500 workers involved in the shoe slipper and handbag industry in Istanbul [3] The working conditions, including the mode of benzene exposure are described elsewhere [3, 6, 7] In Istanbul, during the period from 1967 to 1974, 33 leukemic individuals chronically exposed to benzene[1] were found With the exception of 2 (1 furniture worker, 1 working in a printing shop), all were shoe workers Twenty six of these patients were described elsewhere [3–5] In 1974, 8 new leukemic patients with chronic exposure to benzene were studied in the Internal Clinic of Istanbul Medical School The concentrations of benzene in their working environment measured by a Drager-Spürgerät Multi Gas Detector Mod 21 23, Lubek, were recorded to reach 210 or, rarely, 640 ppm when benzene containing adhesives were used

## Results

Some clinical and hematological findings and the duration of exposure are given in tables I and II

*Types of leukemia* The comparison of the distribution of the types of leukemia found in these 34 individuals chronically exposed to benzene and those of 50 non-exposed leukemic patients is given in table III Similarly, the ages of these non-exposed leukemic individuals who have been studied in our clinic during the last 1½ years varied between 16 and 60 years with a mean of 37 7 years According to our results, acute myeloblastic leukemia seems to be the most frequent variety of this malignancy following exposure to benzene Then comes preleukemia, erythroleukemia and acute lymphoblastic leukemia The criteria for the diagnosis of preleukemia were similar to those of WINTROBE et al [20] (1) the presence of refractory anemia, mostly hypochromic or pancytopenia, (2) the percentages of myeloblasts in the bone marrow within the normal range or slightly increased, and (3) the presence of a few blast cells, mostly myeloblasts, in the peripheral blood film

*Pancytopenic period* A preceding pancytopenic period was present in 23 5% of all cases It was noted in eight patients, three with acute myeloblastic leukemia, three with preleukemia, one with acute monocytic leukemia and one with acute erythroleukemia Preceding pancytopenic period was particularly present in 50% of preleukemic patients The interval between the onset of leukemia and that of pancytopenic period varied from 6 months to 6 years It was the shortest in case 5 and the longest in

[1] One of the 34 leukemic patients was exposed to benzene outside of Istanbul

*Table I* Clinical, hematological and bone marrow findings in 8 new leukemic individuals through c exposure to benzene

| | Cases | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Age, years | 36 | 53 | 18 | 41 | 21 | 45 | 28 |
| Duration of exposure, years | 7 | 20 | 1–2 | 4–12 | 5 | 8 | 1½ |
| Pancytopenic period | + | – | – | + | + | – | – |
| Type of leukemia | AEL | AEL | ALL | PL | PL | CML | AML |
| Hematological findings | | | | | | | |
| RBC × 10⁶/µl | 1.98 | 1.41 | 2.60 | 1.96 | 2.00 | 4.00 | 2.70 |
| Hb, g% | 3.9 | 3.6 | 7.3 | 5.4 | 4 | 10.1 | 8 |
| WBC/µl | 2,500 | 5,600 | 39,000 | 2,800 | 5,600 | 331,000 | 13,400 |
| Reticulocytes, % | 2.8 | 1 | 1 | 1.5 | 1 | 1 | 2.8 |
| Platelets, × 10³/µl | 10.0 | 15.0 | 10.0 | 115 | 10.0 | 300 | 90.0 |
| Hematocrit, % | 16 | 14 | 22 | 18 | 14 | 41 | 25 |
| Myeloblasts, % | 0 | 0 | 0 | 6 | 10 | 1 | 0 |
| Lymphoblasts, % | 0 | 0 | 60 | 0 | 0 | 0 | 0 |
| Myelocytes % | 0 | 0 | 0 | 0 | 0 | 25 | 0 |
| Monocytes, % | 3 | 2 | 1 | 0 | 2 | 0 | 91 |
| Nucleated red blood cells/100 WBC | 11 | 3 | 0 | 0 | 4 | 1 | 1 |
| Bone marrow findings | | | | | | | |
| Myeloblasts, % | 12 | 40 | | 5 | 2[1] | | 4 |
| Monocytes % | 0 | 0 | | 0 | 0 | | 66 |
| Megaloblasts % | 12 | 0 | | 0 | 1 | | 0 |
| Myeloid series, % | 35 | 48 | | 69 | 46 | | 13 |
| Erythroid series, % | 55 | 43.5 | | 2 | 33 | | 14 |

AEL = Acute erythroleukemia, ALL = acute lymphoblastic leukemia, PL = preleukemia, CML = c myeloid leukemia, AML = acute monocytic leukemia, + = present, – = absent.

[1] These findings were delivered by the bone marrow puncture performed in the preceding pancyt period. Two other bone marrow punctures performed during the leukemic phase yielded no mater

case 4, six months and six years respectively. Usually, the clinical and hematological findings of pancytopenia improved considerably or even disappeared completely. But despite this improvement, the findings of leukemia developed later, following a period of variable duration.

*Incidence of leukemia.* During the period of 8 years, 31[2] patients with

[2] Three leukemic individuals were not included in the study due to the profession in 2, one being a furniture worker and the second working in a printing shop. The third individual was not included because his working place was outside of Istanbul.

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* One of the 34 leukemic patients was exposed to benzene outside of Istanbul

*Table IV* Annual number of leukemic shoe-workers in Istanbul between 1967 and 1974

| Years | Number of leukemic shoe-workers | Years | Number of leukemic shoe-workers |
|---|---|---|---|
| 1967 | 1 | 1971 | 6 |
| 1968 | 1 | 1972 | 5 |
| 1969 | 3 | 1973 | 7 |
| 1970 | 4 | 1974 | 4 |

acute leukemia, one lymphoblastic and the other myeloblastic. This family was described elsewhere [5] The third patient, case 8 of the series presented here had acute erythroleukemia His father, a 65-year-old shoe-worker with a long history of benzene exposure died nearly 1 year ago in a hospital in Istanbul with the diagnosis of myelosclerosis But re-evaluation of the case report showed that this patient possibly had an acute leukemia of unidentified type The disease showed an acute course with severe gastrointestinal bleedings and profound anemia and there was no hepatosplenomegaly Four sternal punctures yielded no material

*Some contributary factors for the development of leukemia with a preceding pancytopenic period* In 6 of 8 new patients with acute leukemia, corticosteroids were used during the course of pancytopenic period varying from some months to several years In these pancytopenic patients, acute leukemia or preleukemia developed with an interval of 6 months to 6 years In addition to that, re-exposure to benzene was also noted in 4 of these 6 patients

## *Discussion*

Since the first case report of leukemia due to chronic exposure to benzene appeared [17], numerous leukemic patients with chronic exposure to this chemical were described As an example, Browning [9] collected 61 cases of leukemia with chronic benzene poisoning from the literature till 1965 More than that appeared in the literature since that time The establishment of a marked and statistically significant increase in the incidence of leukemia among shoe workers chronically exposed to benzene by this study and previous works [3] provided final evidence of the leukemogenic effect of benzene in man

*Types of leukemia* Thirty-three of 34 leukemic individuals of this series had various types of acute leukemia Interestingly, there was only 1 case of chronic leukemia, myeloid in type According to this study, acute myeloblastic leukemia seems to be the most frequently encountered type of leukemia following chronic exposure to benzene This finding is in accordance with those of VIGLIANI and SAITA [19] In this study a striking increase in the number of the patients with preleukemia (6 cases, 17 6%) and acute erythroleukemia (6 patients, 17 6%) was noted In this respect, with small differences, the frequency of various morphologic types of acute leukemia in our series was similar to that of GOGUEL *et al* [13], obtained in 23 workers with acute leukemia due to chronic exposure to benzene As noted above, in our series only 1 case of chronic myeloid leukemia was present In contrast to our results, there are several reports describing the occurrence of chronic myeloid or even chronic lymphoid leukemia in the patients with chronic benzene poisoning BROWNING [9] collected 21 cases of chronic myeloid and 6 cases of chronic lymphoid leukemia among 61 leukemic individuals with chronic benzene exposure Furthermore, in 1967 GOGUEL *et al* [13] noted that there were 16 cases with chronic myeloid and 8 with chronic lymphoid leukemia in their series of leukemic patients with exposure to this chemical Therefore, it is difficult to explain the significant difference existing between the distribution of the types of leukemia due to chronic benzene poisoning established in our series and in the group arranged by BROWNING [9] and that of GOGUEL *et al* [13] On the other hand, if we compare the types of leukemia occurring in 34 exposed individuals and 50 non-exposed persons a striking difference can be seen between these two groups of leukemic patients This result is in contrast to that observed among heavily irradiated survivors in Hiroshima and Nagasaki [8, 14–16] The occurrence of chronic myeloid leukemia was frequent among these groups of survivors Furthermore, a small but significant difference was found concerning the incidence of chronic myeloid leukemia in atomic bomb survivors in Hiroshima and Nagasaki, 33 and 18%, respectively [15] This difference in the incidence rate of chronic myeloid leukemia was found to be related to the dose and the nature of the radiation [15] On the other hand, the almost absence of chronic lymphoid leukemia among these groups of survivors of atomic bomb was explained by the infrequent occurrence of chronic lymphoid leukemia in Japan [16] But in Turkish people, the distribution of the types of leukemia is almost similar to that observed in Western countries Therefore, the absence of chronic lymphoid leukemia and the rarity of

chronic myeloid leukemia in our series is significant. On the other hand, our results are somewhat similar to those of COURT BROWN and DOLL [11, 12] obtained in 50 patients receiving partial body irradiation for treatment of ankylosing spondylitis. In this study, 38 out of 50 cases had acute leukemia and only 8 patients with chronic forms, 7 myeloid and 1 lymphoid, have been found. According to this study, the data in the remaining cases were insufficient to determine the clinical type of this disease. Furthermore, there are numerous cases of leukemia following radio iodine treatment [1, 2, 10, 18]. With the exception of 2 examples of chronic myeloid leukemia [2, 18] all patients had acute leukemia.

The foregoing data show that the distribution of the types of leukemia following exposure to benzene varied significantly between studies [3, 9, 13, 19]. A certain relationship between the duration of exposure and the types of leukemia was noted in the present study (table II).

Finally, the role of genetic predisposition was evident in 3 of our leukemic patients in 2 families. In the first family described previously elsewhere [5], uncle and paternal nephew had acute leukemia, one lymphoblastic, the other myeloblastic. In the second family, the father had acute leukemia of unidentified type and his son had acute erythroleukemia. The occurrence of leukemia in these two families was more than a mere coincidence. Therefore, in our opinion, some genetic factors triggered by chronic exposure to benzene played a role in these two families with acute leukemia.

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# Different Composition and Mitotic Activity of the Haemopoietic Tissue in Bone Marrow, Spleen and Liver in Chronic Myeloid Leukaemia

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*Key Words.* Bone marrow · Extramedullary haemopoiesis · Liver · Myeloid leukaemia · Spleen.

*Abstract.* Aspirates from bone marrow, spleen and liver were analyzed in 15 untreated patients with chronic myeloid leukaemia (CML). The proportion of erythroblasts was higher in the spleen and liver than in the bone marrow. An increased frequency of basophilic leucocytes was recorded in the extramedullary sites compared to the bone marrow. In the liver aspirates a considerable admixture of lymphocytes was found. The mitotic indices of the granulopoietic and erythropoietic precursor cells were lower in the spleen and liver than in the bone marrow. The results indicate that the composition and proliferative activity of the haemopoietic tissue differ in bone marrow and extramedullary sites in CML. The discrepancies may be due to differences in the microenvironment for the haemopoietic cells or to a selective accumulation of cells with a low mitotic activity in extramedullary sites. Since the final blastic transformation of CML may originate outside the bone marrow, further studies of the extramedullary haemopoietic tissue may be worthwhile.

The chronic phase of chronic myeloid leukaemia (CML) is characterized by a successive increase of intra- and extramedullary haemopoietic cells. Although a reduction of the blood forming tissue may be temporarily achieved due to treatment, the final blastic transformation usually develops in patients with considerable hepatomegaly and splenomegaly and whose bone marrow is overcrowded with cells.

Practically no information is available on the composition and function of the haemopoietic tissue in the liver in CML but there are several indications that the blood forming tissue of the spleen and the bone marrow may differ in various respects. Thus, in the chronic phase of the disease, discrepancies in the composition and kinetic properties of the granulopoietic tissue have been demonstrated [4, 5, 7, 21] and differences between intramedullary and splenic erythropoietic tissue have also been found [24]. The

significance of these differences is far from clear but since it has been demonstrated that morphologic or cytogenetic signs of the terminal blastic crisis may be detected in the spleen [19–22], in lymph nodes [10, 12, 15, 17, 28] as well as in other extramedullary sites [14, 16] prior to such changes in the bone marrow, it may be worth-while to study the extramedullary blood forming tissue more extensively in CML

The present investigation was undertaken in order to compare the composition of the haemopoietic tissue in liver and spleen to that of the bone marrow in untreated patients with CML In addition the mitotic activity of the granulocytic and erythroid precursors was compared in the three organs

## *Material and Methods*

*Patients* 15 untreated patients with a recent diagnosis of CML were examined The WBC counts were 76,000–311 000 No patient was in blastic crisis

*Morphological investigations* Bone marrow was obtained through sternal puncture and material from the liver and spleen through fine-needle aspiration biopsy as described by SÖDERSTRÖM [26] Aspirates from the three organs were obtained on the same occasion and smears were stained with May Grünwald Giemsa Through examination of 1,000–3,000 (mean 1,100) nucleated cells the percentages of granulocytic cells at different stages of maturation, erythroblasts, basophilic leucocytes and lymphocytes were determined Neither cells belonging to the liver parenchyma nor splenic lymphocytes or pulp cells were included in these calculations A mitotic index of the granulopoietic precursor cells (myeloblasts + promyelocytes + myelocytes) was determined in each smear through examination of 1,600–7,500 (mean 3 800) such cells and a mitotic index of the erythroid precursor cells through examination of 500–1,800 (mean 950) erythroblasts At the same time the erythroblasts were morphologically classified Proerythroblasts and basophilic erythroblasts were then pooled into one group designated basophilic erythroblasts

*Statistics* The Wilcoxon matched pairs signed ranks test was used to assert significance of the results

## *Results*

*Composition of intra- and extramedullary haemopoietic tissue* The median proportion of erythroid precursors in the bone marrow was 3 6% In the spleen the corresponding figure was 7 5% which is significantly higher ($p<0\ 02$) A higher median percentage of erythroblasts, 6 7%, was also recorded in the liver although the difference between the bone marrow and the liver aspirates was not fully significant ($p<0\ 06$) (fig 1)

In the bone marrow on an average 3 0% of the cells were basophilic leucocytes compared to 4 7% in the spleen and 4 8% in the liver The

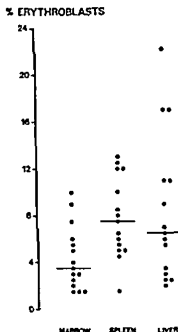



*Fig 1* Percentage of erythroblasts within the haemopoietic tissue of bone marrow spleen and liver in 15 patients with CML. Median values are indicated

increase of basophilic cells was significant ($p<0.01$) in both spleen and liver when compared to the bone marrow (fig 2)

The percentage of lymphocytes was 0.3–3.5% (median 1.3%) in the bone marrow and 3.6–17.1% (median 6.4%) in the liver The difference is significant ($p<0.01$)

*Mitotic activity* The median mitotic index of the granulopoietic precursor cells was 1.07% in the bone marrow, 0.70% in the spleen and 0.64% in the liver (fig 3) The mitotic indices in both extramedullary sites were significantly lower than in the bone marrow ($p<0.01$) There were no indications that the discrepancies in mitotic activity were due to the presence of different amounts of mature neutrophils known to produce substances that interfere with the proliferation of granulopoietic precursors [23] Thus the percentage of mature neutrophils was on an average 35.0% in the bone marrow, 38.3% in the liver and 29.5% in the spleen

The mitotic indices of the erythroblasts were likewise significantly lower ($p<0.01$) in the spleen and liver than in the bone marrow the median values

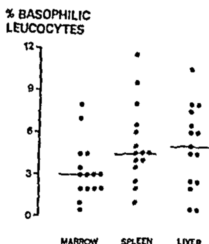



*Fig 2* Percentage of basophilic leucocytes in bone marrow, spleen and liver

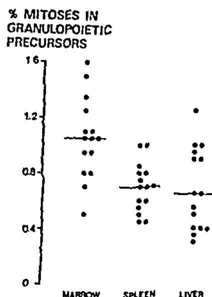



*Fig 3* Mitotic indices of granulopoietic precursor cells in bone marrow, spleen and liver

being 2.5, 2.6 and 3.5%, respectively (fig 4) The higher indices in the bone marrow were not caused by an unduly large proportion of early erythroid cells known to have higher mitotic indices than the more mature erythroblasts Thus the average proportion of basophilic erythroblasts within the erythropoietic pool in the marrow was 37.8% compared to 38.4% in the spleen and 40.0% in the liver

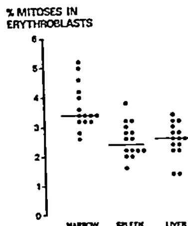



*Fig 4* Mitotic indices of the erythroblasts in bone marrow, spleen and liver

## *Discussion*

The present data indicate that several characteristics are common to extramedullary haemopoietic tissue whether situated in the spleen or the liver in CML Furthermore, in several respects the extramedullary tissue was found to differ from the bone marrow It has previously been demonstrated that in CML the proportion of erythroblasts within the haemopoietic tissue is larger in the spleen than in the bone marrow and that the increasing splenomegaly during the course of the disease therefore means a considerable contribution of erythroid precursors [24] The present data indicate that the extramedullary tissue in the liver is also relatively rich in erythroid cells It is known that haemodilution and sequestration of red cells in the enlarged spleen contribute to the development of anaemia in CML [2, 3, 9] The low mitotic activity of the spleen and liver erythroblasts may help to explain why the large amount of extramedullary erythropoietic tissue in advanced CML obviously fails to compensate factors that promote the development of anaemia

A low mitotic activity was also found in the granulopoietic precursors in spleen and liver An accumulation in the bone marrow of early granulopoietic precursor cells with a low mitotic activity at diagnosis of CML has been found to indicate a serious prognosis with a short life span, i e the patient may be near the final blastic crisis [25] Furthermore, in Ph¹-chromosome-negative CML, known to generally run a more rapid malignant

course than Ph¹-chromosome-positive CML [11], the mitotic indices of the granulopoietic precursor cells have been found to be extraordinarily low [6]. It may therefore be that a low mitotic activity of granulopoietic precursors is a sign of 'leukaemicness' in CML and if so the prevalence of precursors with a low mitotic activity found in the spleen and liver may indicate that these organs lodge cells with even more malignant properties than the bone marrow. In this context it is of interest to note that the mitotic indices of the granulopoietic precursors of the spleen and liver in the present cases were often as low as those generally found in bone marrow blast cells of a group of patients with acute myeloid leukaemia recently studied by us [8]. Furthermore, in two CML patients karyotypic changes characteristic of the blastic transformation have been found in the spleen before they could be detected in the bone marrow, which may likewise indicate an increased tendency to malignant behaviour of extramedullary haemopoietic tissue in CML [19, 20].

The cause of the characteristic increase of basophilic leucocytes in chronic myeloproliferative disorders is unknown. In the present CML patients the basophilic leucocytes were significantly more common within the haemopoietic tissue of the spleen and liver than in the bone marrow. It is known that basophilic leucocytes will increase in tissues where cell-
[illegible] ...that the
[illegible]
cytes [27]. It is therefore possible that the overrepresentation of basophilic leucocytes in the liver and spleen in CML reflects a cell-mediated immunological response against leukaemic cells occurring in these organs. If haemopoietic tissue in spleen and liver is more prone to malignant transformation than the bone marrow, it is conceivable that immune response would be more pronounced in the extramedullary sites. Kaur *et al.* [13] have demonstrated an increase of T lymphocytes in the spleen in CML which is in agreement with such an interpretation, since their findings suggest that cell-mediated immune reactions against leukaemic cells may take place in that organ. The admixture of lymphocytes was significantly higher in the liver specimens than in the bone marrow of our patients. It is known that liver biopsy specimens from normals contain considerable numbers of lymphocytes [18, 26] and it is therefore uncertain whether the relatively high proportions of these cells found in the liver represented normally occurring lymphocytes or were caused by an accumulation of lymphoid cells in the liver. Nevertheless the results indicate the presence of a considerable amount of lymphocytes in the liver in CML, and it cannot be ruled out that these cells may exert immune reactions similar to those in the spleen.

Even if the present results together with results of previous cytogenetic investigations might suggest that extramedullary tissue in CML is more prone to malignant transformation than bone marrow tissue, the reason for this discrepancy remains obscure It is, however, apparent that the environment for the haemopoietic tissue is different in bone marrow and extramedullary sites It is possible that at initiation of CML the haemopoietic cells are programmed for a development ending up in the blastic transformation and that an unfavourable microenvironment for the blood-forming cells in extramedullary sites may promote this development Another possibility is that during the chronic phase of CML circulating cells with potential malignant properties are more or less selectively trapped in extramedullary organs and there may give rise to immune reactions and undergo the final blastic transformation

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# Leukozytenlipide bei reifzelligen Leukämien

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*Key Words* Leukemic leukocytes Leukocyte lipids Lipids in leukemia

*Abstract* The total lipid content and the lipid pattern of normal and leukemic leukocytes showed no significant difference except the fact, that lymphocytes of chronic lymphatic leukemia have a lower content of triglycerides and sphingomye line Lecithin of leukemic lymphocytes contained more palmitinic acid and oleic acid and a lower level of stearic acid than the same fraction in normal lymphocytes. The lipid pattern of normal and leukemic granulocytes was identical

Da die Lipide einen wesentlichen Bestandteil der Zellmembran darstellen, wurden lipidanalytische Untersuchungen auch an Tumorzellen wiederholt vorgenommen, zumal bestimmte Tumorzelleigenschaften, wie die Antigenitat, Kontaktinhibition, Adhäsivität, Permeabilität, Oberflächenladung u a , gegenuber normalen Zellen verandert sind und damit auf Abweichungen im Zellmembranaufbau hinweisen [1, 3, 6, 12, 17–20] Art und Zustandekommen dieser Veranderungen, ihr Zusammenhang mit der neoplastischen Umwandlung und die funktionellen Auswirkungen auf die Zelle sind im einzelnen meist unklar

Wegen der Möglichkeit, weitgehend reine Zellanreicherungen zu gewinnen, sind Blutzellen für vergleichende Untersuchungen am Menschen besonders geeignet

Bereits vor Jahrzehnten erfolgten Gegenüberstellungen des Lipidgehaltes normaler und leukämischer Leukozyten Erst seit etwa 15 Jahren sind jedoch aus methodischen Gründen reproduzierbare Ergebnisse

möglich In diesem Zeitraum wurden vor allem von KIM *et al* [8], SCHWANDT *et al* [15, 16] und GOTTFRIED [4, 5] vergleichende Untersuchungen leukämischer Leukozyten durchgeführt Nur GOTTFRIED zog zum Vergleich gereinigte normale Leukozytenpopulationen heran, während die anderen Untersucher Gesamtleukozytenanreicherungen verwandten Unter Bezug auf die Zellzahl fand GOTTFRIED bei allen neoplastischen Leukozyten, aber auch bei langfristig kultivierten Normalzellen gleichartige Veränderungen des Zellipidgehaltes, nämlich Verminderung des Gesamtlipids, Verminderung des Cholesterins, relative Vermehrung der Phospholipide Innerhalb der Phospholipide waren Sphingomyelin vermindert und Phosphatidylcholin vermehrt Diese Veränderungen wurden von ihm als unspezifisches Zeichen der Unreife gedeutet und nicht mit der neoplastischen Umwandlung in Zusammenhang gebracht

Die vorliegende Arbeit untersucht Lipidgehalt, Lipidverteilung und Fettsäuremuster von Leukozyten bei reifzelligen Leukosen Unter Benutzung der Eiweissmenge als Bezugsgrosse konnten bei der Gegenüberstellung mit normalen gereinigten Leukozytenpopulationen sichere Abweichungen nur bei der lymphatischen Leukose nachgewiesen werden Die Differenzen zwischen normalen und leukämischen Granulozyten waren meist nur geringfügig und statistisch nicht zu sichern

## *Material und Methodik*

Ausgangsmaterial waren jeweils 40 ml frisches Zitratblut Die Isolierung von Lymphozyten und Granulozyten erfolgte nach der von OTTO und SCHMID [13 14] beschriebenen Methodik Anstelle von Uromiro® wurde Visotrast® auf das erforderliche spezifische Gewicht eingestellt

Für die Kontrollgruppe wurde Blut Blutgesunder aufbereitet, wobei Lymphozyten bzw Granulozytenanreicherungen von jeweils 5–10 Patienten zusammengenommen und als eine *Einzelprobe verarbeitet wurden Die Lipidanalyse* der Leukosezellen erfolgte aus Einzelblutproben von Patienten die keine zytostatische Behandlung *erhielten* und eine Leukozytenzahl von mindestens 50 000/µl aufwiesen Die Lipidextraktion erfolgte nach Folch Die Homogenisierung der Zellen wurde in Chloroform Methanol (2 1) mit dem Ultraturrax vorgenommen Nach Filtration und Einengung wurden die Lipide dünnschichtchromatographisch getrennt, Neu*tralfette im* System Petroläther/Äther/Eisessig Phosphatide im System Chloroform/Methanol/Wasser

Nach Abtragen der mit Jod bzw Rhodamin gefärbten Banden wurden die Fraktionen einzeln bestimmt Cholesterin und Cholesterinester nach BEUKERS *et al* [2] Triglyzeride mit der Sulphophosphovanillinreaktion nach ZÖLLNER und EBER

HAGEN [21], die Phosphatide nach Veraschung mit Perchlorsäure als Orthophosphat nach MARTIN und DOTY [10] Alle Methoden wurden auf die Anwendung auf Kieselgel adaptiert, eine ausführliche Darstellung erfolgte bereits [7] Als Bezugsgrösse diente der Gehalt an Eiweiss, die Eiweissbestimmung wurde nach LOWRY *et al* [9] vorgenommen

An normalen und leukämischen Lymphozyten wurde eine Fettsäureanalytik der Phospholipide durchgeführt. Hier wurde sämtlichen bei der Phospholipidextraktion und trennung verwendeten Lösungsmitteln das Antioxydans BHT zugesetzt. Die dünnschichtchromatographische Phospholipidtrennung erfolgte in der Modifikation einer Zweischrittrennung Als erstes Laufmittel wurde Chloroform/Methanol/Wasser (130-50 8), als zweites Laufmittel Petroläther/Äther (180-20) verwandt.

Nach Anfärben der Lipidbanden mit 2,7 Difluoreszein wurde die Kieselgelzone abgetragen und unter $N_2$ ampulliert. Die Umesterung der Fettsäuren zu den Methylestern erfolgte mit methanolischem $BF_3$ entsprechend den Angaben von MORRISON und SMITH [11]

Die gaschromatographische Trennung wurde mit einem Gaschromatographen der Firma Varian Aerograph, Modell 2100, ausgerüstet mit einem Flammenionisationsdetektor und einem elektronischen Integrator (Modell 475) der gleichen Firma durchgeführt

Die Säulenfüllung erfolgte mit 10% EGSS-X auf Gaschrom-Q-100-120 mesh Für die Identifizierung der einzelnen Fettsäuren wurde isotherm bei 180 °C gearbeitet, für die quantitative Bestimmung wurde die Temperatur programmiert von 150-190 °C mit einer Heizrate von 1 °C/min Die quantitative Auswertung wurde mit den NIH Testsubstanzen kontrolliert und gewährleistet. Die statistische Berechnung erfolgte nach Prüfung der Normalverteilung mit dem t Test.

## *Ergebnisse*

Der mittlere Lipidgehalt pro Milligramm Eiweiss lag nach Addition der Einzelfraktionen bei normalen Lymphozyten bei 421,6 nmol, bei leukämischen Lymphozyten bei 440,7 nmol Ein statistisch sicherer Unterschied bestand zwischen beiden Werten nicht. Normale Granulozyten enthielten 693,9 ± 121,7 nmol Lipid/mg Eiweiss, leukämische Granulozyten 482 ± 112,2 nmol/mg Eiweiss. Der Unterschied zwischen beiden Leukozytengruppen war nicht signifikant (t = 1,4)

Der Phospholipidanteil am Gesamtlipid bewegte sich zwischen 68 und 77%. Es ergaben sich keine signifikanten Abweichungen zwischen normalen und leukämischen Zellen

An Neutralfetten enthielten normale Leukozyten prozentual mehr Triglyzeride als leukämische Dieser Unterschied war jedoch nur bei den Lymphozyten signifikant (Tabelle I)

[illegible] und Granulozyten

| [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
|---|---|---|---|---|---|---|
| | | s | x̄ | s | x̄ | s |
| [illegible] | [illegible] | 12,1 | [illegible] | [illegible] | [illegible] | [illegible] |
| [illegible] | [illegible] | 9,1 | [illegible] | [illegible] | [illegible] | [illegible] |
| [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | 36,3 | [illegible] |

[illegible] zu den [illegible] in [illegible] und Granulozyten

| [illegible] | PC | | PÄ | | PS–PG | |
|---|---|---|---|---|---|---|
| | x̄ | | | s | x̄ | s |
| [illegible] | | | | | [illegible] | 2,3 |
| | 3,2 | | | | | |
| | 9,9 | | | | | |
| | 5,2 | | | | | |

Sphingomy [illegible]

= Phospha [illegible]

[illegible]

| Fettsäuren | Normal | | Leukämisch | |
|---|---|---|---|---|
| | x̄ | s | x̄ | s |
| *Phosphatidylcholin* | | | | |
| 16 0 | 14,0 | 7,2 | 41,9 | 1,87[1] |
| 18 0 | 53,8 | 1,14 | 20,1 | 2,63[1] |
| 18 1 | 13,9 | 1,9 | 20,6 | 1,48[1] |
| 18 2 | 3,5 | 0 | 4,27 | 2,94 |
| 20 0 | 1,78 | 0,78 | 0,63 | 0,11 |
| 20 4 | 4,7 | 0 | 3,65 | 2,6 |
| *Phosphatidyläthanolamin* | | | | |
| 16 0 | 7,16 | 1,5 | 12,2 | |
| 18 0 | 42,7 | 20,6 | 45,0 | |
| 18 1 | 9,49 | 4 67 | 17,3 | |
| 18 2 | 4,36 | 2,0 | 1,85[2] | |
| 20 0 | 1,36 | 1,97 | 0,52 | |
| 20 4 | 3,09 | 1,58 | 3,59 | |
| *Sphingomyelin* | | | | |
| 16 0 | 14,1 | 2,2 | 16,4 | |
| 18 0 | 49,7 | 0 | 48,5 | |
| 18 1 | 4,96 | 1,4 | 9,7[2] | |
| 18 2 | 10,1 | 2,6 | 0,6 | |
| 20 0 | 1,0 | 0,4 | 7,8 | |
| 20 4 | 1,2 | 0,4 | 0,5 | |

[1] $p<0{,}05$
[2] Einzelbestimmungen

Normale und leukämische Lymphozyten wiesen eine unterschiedliche Phospholipidverteilung auf. Leukämische Lymphozyten enthielten signifikant weniger Sphingomyelin als normale. Eine entsprechende Lezithinvermehrung war statistisch *nicht zu sichern. Gleichartige* Veränderungen konnten an den Granulozyten nicht nachgewiesen werden (Tabelle II)

Wegen der Unterschiede der Phospholipidverteilung wurde bei normalen und leukämischen Lymphozyten eine Fettsäureanalyse durchgeführt Dabei enthielt das Lezithin leukämischer Lymphozyten

signifikant mehr Palmitin- und Ölsäure, die gleiche Fraktion normaler Lymphozyten mehr Stearinsäure (Tabelle III).

*Diskussion*

Aus unseren Befunden ist die Schlussfolgerung zu ziehen, dass in Relation zum Eiweissgehalt keine signifikanten Veränderungen im Gesamtlipidgehalt bei Vergleich normaler und leukämischer Zellen nachweisbar sind und die absoluten mittleren Lipidmengen, bezogen pro Milligramm Eiweiss bei normalen und leukämischen Lymphozyten etwa gleich hoch, bei leukämischen Granulozyten leicht, jedoch nicht signifikant vermindert sind. Normale Granulozyten enthalten am meisten, normale Lymphozyten am wenigsten Lipid. Vergleiche zwischen leukämischen Lymphozyten und normalen Gesamtleukozytenanreicherungen, die vorwiegend Granulozyten enthalten, sind damit nicht möglich.

Bis auf die relative Sphingomyelinverminderung, die auch wir bei leukämischen Lymphozyten nachweisen konnten – bei leukämischen Granulozyten bestanden ähnliche Veränderungen nicht – weichen unsere Befunde von denen von GOTTFRIED [4, 5] ab. Dies möchten wir auf unterschiedliche Bezugsgrössen zurückführen. Ein Zugrundelegen der Zellzahl kann z.B. Zellgrossen- und Strukturveränderungen nicht berücksichtigen, ermöglicht dagegen eine Aussage über den Lipidgehalt der Einzelzelle.

Der Bezug auf Eiweiss vergleicht mit einem biochemischen Parameter, der mit gewissen Einschränkungen als Mass der Stoffwechselaktivität gesehen werden kann.

Das Fettsäuremuster der Phospholipide bestimmt weitgehend deren physiologische Eigenschaften und scheint für wichtige Membraneigenschaften (Aktivität membranständiger Enzyme, Permeabilität u.a.) von ausschlaggebender Bedeutung zu sein. Die zur Verfügung stehenden Materialmengen erlaubten lediglich Einzelbestimmungen der Fettsäuren von Phosphatidyläthanolamin und Sphingomyelin leukämischer Lymphozyten. Die ersichtlichen Unterschiede im Phosphatidyläthanolamin bei 16:0, 18:1 und 18:2, in Sphingomyelin bei 16:0, 18:1, 18:2 und 20:2 wurden daher statistisch nicht abgesichert. Der Vergleich der Fettsäuremuster der Phosphatidhauptfraktion Phosphatidylcholin normaler und leukämischer Lymphozyten ergab signifikante Veränderungen bei Palmitin-, Stearin- und Ölsäure. Da schon im Normalfall die Funk-

tion der einzelnen Fettsauren unklar ist, kann eine Interpretation dieser Abweichungen nicht erfolgen oder müsste zwangsläufig spekulativ sein

## *Zusammenfassung*

Vergleichende Untersuchungen an normalen und leukämischen Leukozyten ergaben keine signifikanten Veränderungen des Gesamtlipidgehalts Lymphozyten von reifzelliger lymphatischer Leukämie enthielten im Vergleich mit normalen Lymphozyten geringere Anteile Triglyzerid und Sphingomyelin Das Lezithin leukämischer Lymphozyten enthielt mehr Palmitin und Ölsäure und weniger Stearinsäure als die gleiche Phosphatidfraktion normaler Lymphozyten Die Lipidverteilung normaler und leukämischer Granulozyten war nicht signifikant unterschiedlich

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# Relationship between the Concentration of Neutrophils and Monocytes in Venous Blood

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*Key Words* Chronic granulocytic leukaemia Exercise Marginated leucocytes Monocytes Myeloproliferative disorders Neutrophils Pregnancy

*Abstract* A striking linear relationship was found between the logarithms of the medians of the circulating neutrophil (granulocyte plus metamyelocyte) counts and the monocyte counts in 5 groups of individuals studied. These groups consisted of (1) 100 normal adult males, (2) 100 normal non pregnant females, (3) 50 females in the last trimester of pregnancy (4) 15 patients with a persistent neutrophil leucocytosis and (5) 20 patients with polycythaemia rubra vera, essential thrombocythaemia or myelofibrosis. A different relationship appeared to exist between the circulating neutrophil and monocyte counts in a group of 16 patients with chronic granulocytic leukaemia

In a recent study, TWOMEY *et al* [7] found a linear relationship between the logarithm of the concentration of circulating neutrophils and the logarithm of the concentration of circulating monocytes in a group of individuals consisting of 40 normal adults and 22 patients with aplastic anaemia. This observation is of considerable theoretical interest as it supports other data indicating a close relationship between neutrophil granulocytopoiesis and monocytopoiesis. Thus several studies have shown that a single progenitor cell can give rise to many monocytes, macrophages and neutrophils when grown in a suitable semi-solid medium in the presence of colony-stimulating factor (CSF). Furthermore, both monocytes and macrophages have been shown to be potent sources of CSF. The present study was undertaken to determine the relationship, if any, between the concentration of circulating neutrophils and monocytes in individuals with an elevated neutrophil count. This information would be of import

ance in formulating any hypotheses regarding the mechanism regulating human neutrophil granulocytopoiesis and monocytopoiesis At present, the regulation of both these cell renewal systems is incompletely understood

## *Patients and Methods*

Six groups of individuals were studied These consisted of (1) 100 normal adult males, (2) 100 normal adult females (mainly pre menopausal) (3) 50 females during the last trimester of pregnancy, (4) 15 patients with a reactive neutrophil leucocytosis in whom the neutrophil count had been elevated for a minimum of 7 days (5) 16 patients with chronic granulocytic leukaemia (Ph' positive) and (6) a group made up of 10 patients with polycythaemia rubra vera, 5 patients with essential thrombocythaemia and 5 patients with myelofibrosis The diagnoses in the patients with a reactive neutrophil leucocytosis included pyogenic abscesses, bronchopneumonia Crohn s disease and ulcerative colitis The data on the 100 normal males and 100 non pregnant females were taken from an earlier study of the normal neutrophil count, in which a correlation was observed between the circulating neutrophil and monocyte counts [2]

The concentrations of circulating neutrophils neutrophil metamyelocytes and monocytes were calculated from the total nucleated cell count determined on a Coulter counter, model S and a 500-cell differential leucocyte count Only a single sample of venous blood was studied per individual, except in one healthy pre-menopausal adult female In the latter, 80 samples were studied at regular intervals over an 8 month period Except in this subject in whom all the blood counts were performed between 9 00 a m and 10 00 a m after a 30-min period of rest, the counts were performed at any time between 9 00 a m and 5 00 p m, without a preceding period of rest

Pre and immediate post-exercise venous blood samples were studied in 11 healthy adults (8 males and 3 females) who were exercised on a bicycle ergometer The males were exercised for 10 min with a work load of 138 W and the females for 6 min with a work load of 69 W The concentration of various cell types in these samples were calculated from the total nucleated cell counts and 2 000-cell differential counts The post exercise cell counts were corrected for changes induced by haemoconcentration, assuming that the degree of haemoconcentration is proportional to the exercise induced change in the haemoglobin concentration

## *Results and Discussion*

A highly significant correlation was found between the logarithm of the concentration of circulating neutrophil granulocytes plus metamyelocytes (log N) and the logarithm of the concentration of circulating monocytes (log M) in both the normal adult males (r = 0 39, m = 0 40,

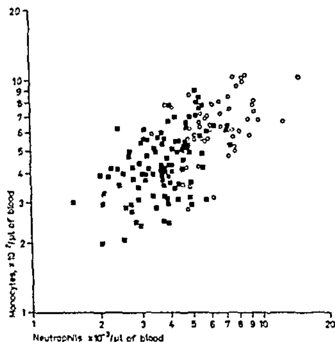



*Fig 1* Relationship between the concentration of neutrophil granulocytes plus metamyelocytes and the concentration of monocytes in the circulation of 100 healthy non pregnant females (■) and 50 females in the last trimester of pregnancy (○)

p<0 001) and the normal adult females (r = 0 45, m = 0 49, p<0 001) A similar correlation was also seen in the group of pregnant females (r = 0 41, m = 0 45, p<0 001) and the regression line for this group was virtually identical to that for the group of non-pregnant females (fig 1) Although all the above-mentioned correlations reached a high degree of statistical significance, the correlations were quite weak; only 15–20% of the observed variation in log M can be accounted for by variation in log N and vice versa This poor correlation was not entirely due to person to-person variations in the relationship between log M and log N or to diurnal variations in cell counts as the correlation between log N and log M in the samples of blood taken between 9 00 a m. and 10 00 a m on 80 different days from a single pre-menopausal woman (r = 0.31) was not significantly different from that in the entire group of non pregnant fem-

*Table I.* Pre- and post-exercise leucocyte counts in 11 normal subjects

| Cell type | Median cell count $\times 10^{-9}/l$ | | Post-exercise/pre-exercise | | Significance of exercise-induced change p |
|---|---|---|---|---|---|
| | pre-exercise | post-exercise[1] | range | median | |
| Neutrophil granulocytes + metamyelocytes | 3,306 | 3,915 | 1.08–1.41 | 1.18 | <0.001 |
| Lymphocytes | 2,094 | 4,006 | 1.67–2.51 | 1.91 | <0.001 |
| Monocytes | 457 | 778 | 1.15–2.54 | 1.70 | <0.001 |
| Eosinophil granulocytes | 105 | 151 | 1.02–2.17 | 1.45 | <0.01 |
| Basophil granulocytes[2] | 35 | 48 | 0.63–3.09 | 1.39 | <0.05 |

[1] Corrected for post-exercise haemoconcentration.
[2] A post exercise increase was found in 9 of the 11 subjects.

ales. As CARTWRIGHT *et al.* [4] have shown that the fraction of the total blood granulocyte pool which circulates may vary between 16 and 99% in resting, normal individuals, it is possible that the weak correlations observed in this study are partly caused by a poor correlation between the sizes of the circulating and total blood granulocyte pools. It is also possible that one or more factors which are independent of the size of the total blood neutrophil or monocyte pool (e.g. oestrogen and progesterone levels in females) affect log N and log M to different extents.

Vigorous exercise, which mobilises cells from the marginated to the circulating leucocyte pool [1], causes a greater increase in the concentration of circulating monocytes than in the concentration of circulating neutrophil granulocytes plus metamyelocytes (table I), indicating that changes in the equilibrium between the marginated and the circulating leucocyte pools affect the correlation between log N and log M. It is of interest that the results shown in table I provide evidence for the existence of fairly large marginated pools of eosinophil and basophil granulocytes. A post-exercise increase in eosinophil and basophil counts was also observed by BELSKY *et al.* [3].

There was no correlation between log N and log M in the groups with (1) a reactive neutrophil leucocytosis ($r = 0.15$, 95% confidence limits, –0.39 to +0.61), (2) polycythaemia vera, myelofibrosis or essential thrombocythaemia ($r = -0.11$, 95% confidence limits, –0.53 to +0.35)

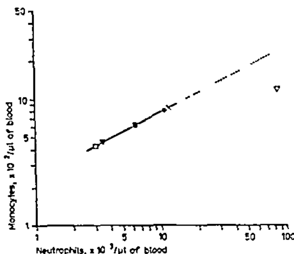



Fig 2 Relationship between the median values for the concentration of circulating neutrophil granulocytes plus metamyelocytes and the concentration of circulating monocytes in the various groups studied. □ = 100 normal males, ○ = 100 non-pregnant, normal females, ■ = 50 pregnant females, ● = 15 patients with a reactive neutrophil leucocytosis, ▽ = 16 patients with chronic granulocytic leukaemia, × = 20 patients with other chronic myeloproliferative disorders, ▼ = a single normal, non pregnant female studied on 80 different days, over an 8 month period

and (3) chronic granulocytic leukaemia (r = −0 12, 95% confidence limits, −0 28 to +0 37) However, the relationship between log N and log M which exists in normal adults is found to apply to the first two of these groups, if the median values for the concentration of circulating neutrophils (granulocytes plus metamyelocytes) and monocytes are considered With the exception of the group of patients with chronic granulocytic leukaemia, there was a striking linear relationship between the logarithms of these median values (fig 2) This implies that in reactive leucocytosis and myeloproliferative disorders (other than chronic granulocytic leukaemia), a weak correlation does exist between log N and log M but that this correlation may not become evident until much larger groups of patients are studied

In chronic granulocytic leukaemia, the relationship between log N and log M is altered Whereas the median neutrophil count is increased 28-fold in comparison with normal males, the median monocyte count is in-

creased only 3 fold (fig 2) The explanation for the 'abnormal' relation ship between log N and log M which occurs in chronic granulocytic leu kaemia is uncertain Three possible explanations are (1) that the *in vitro* colony-forming cells derived from the Ph¹-positive multipotent haemopoietic stem cells respond abnormally to the various regulatory mechanisms which govern the production of neutrophils and mononuclear phag ocytes, (2) that these regulatory mechanisms are disturbed in chronic granulocytic leukaemia and (3) that the linear relationship between log N and log M does not hold when the circulating neutrophil count is very high It must also be mentioned that disturbances in the normal relation ship between log N and log M are not confined to patients with chronic granulocytic leukaemia It has been known for a considerable time that the circulating neutrophil and monocyte counts do not change synchron ously during the development or resolution of a neutrophil leucocytosis [5], nor do they in cyclical neutropenia [6]

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# Increased Proportion of B Lymphocytes in Human Tonsils and Appendices

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*Key Words* Appendix Lymphocytes Tonsils

*Abstract* The percentage of T and B lymphocytes was studied in human tonsils and appendices. An increased percentage of B lymphocytes, 59.1% in tonsils and 54.5% in appendices, was found, whilst the percentage of T lymphocytes was 23.6% and 27.9%, respectively. In the peripheral blood the corresponding figures were 12.5% for B cells and 50% for T cells.

It has been recently shown that there are two major populations of lymphocytes [10, 13] thymus-derived (or T) lymphocytes, involved mainly with cell mediated immunity and bursa-derived (or B) lymphocytes mediating humoral immunity. The reported percentage for T lymphocytes in the peripheral blood varies normally from 40 to 58% [4, 6] while that for B lymphocytes varies from 13 to 34% [9, 13]. In the spleen and the lymph nodes the percentage of T lymphocytes varies from 20 to 36.5% [2, 11] and from 18 to 60% [3, 11] respectively, while that of B lymphocytes varies from 45 to 58% in the spleen [2, 5, 12] and from 21 to 31% in the lymph nodes [8, 11].

In view of the lack of information about T and B cell populations in human tonsils [8] and appendix we thought it worthwhile to study the distribution of T and B lymphocytes in these organs.

## *Material and Methods*

*Lymphocytes.* Lymphocytes were obtained from the peripheral blood of 10 normal subjects, from tonsils of 10 patients undergoing tonsillectomy and from append

ices of 10 patients with appendectomy In 2 cases, normal appendices were removed during hysterectomy

Lymphocytes were separated from the peripheral blood by means of Ficoll Hypaque Lymphocytes from tonsils and appendices were isolated by finely mincing the organs in a small volume of Medium 199 and removing the debris by filtration through fine steel mesh

*Surface immunoglobulins* A direct immunofluorescence method [11] was used by means of fluorescein conjugated polyvalent antiserum (anti IgG $\varkappa$ and $\lambda$, Hyland)

*EAC rosettes* AB Rh+ human red blood cells (HRBC) from heparinized whole blood were washed thrice in normal saline, 2 ml of a 5% HRBC suspension in Hanks' balance salt solution (HBSS) were mixed with equal volume of inactivated rabbit anti HRBC serum (prepared by injecting AB Rh+ HRBC in a rabbit) diluted 1 1000 and the mixture incubated at 37 °C for 1 h The cells were washed twice in veronal buffer saline (VBS) Then 2 ml of fresh mouse serum (as a source of complement) diluted 1 10 in VBS was added to 2 ml of cell suspension and incubated at 37 °C for 1 h The red cells were finally washed twice and adjusted to 0 5% suspension in HBSS (C-coated HRBC)

Of the lymphocyte suspension 0 4 ml containing $1 \times 10^6$ cells were mixed with 0 4 ml of the 0 5% C-coated HRBC suspension and rotated gently for 30 min at room temperature One drop was mounted onto a glass slide, covered by a coverslip and the percentage of lymphocytes binding three or more red cells was determined after counting 300 lymphocytes

*Spontaneous rosette formation* with sheep red blood cells was used to determine T lymphocytes [7]

## *Results*

The results are summarized in the tables I and II The mean percentage of T lymphocytes in the peripheral blood of normal subjects was 50% compared to 23 6 and 27 9% of the tonsils and appendices, respectively The mean percentage of B lymphocytes in the peripheral blood was 12 5% while it was significantly higher in the tonsils (59 1%) and appendices (54 5%) There was no significant difference between the results obtained by the EAC-rosettes and those obtained by the surface-bound immunoglobulins

## *Discussion*

The present observations indicate that human tonsils and appendix contain a large proportion of B lymphocytes, much higher than the peripheral blood On the other hand, the percentage of T lymphocytes in the above organs is significantly lower than in the blood Two possible ex

*Table I* Percentage of B lymphocytes

| | Peripheral blood | | Tonsil | | Appendix | |
|---|---|---|---|---|---|---|
| | EAC | Ig | EAC | Ig | EAC | Ig |
| | 9 | 7 | 54 | 56 | 55 | 57 |
| | 10 | 9 | 56 | 57 | 48 | 52 |
| | 12 | 12 | 50 | 55 | 48 | 50 |
| | 11 | 12 | 58 | 60 | 50 | 50 |
| | 9 | 11 | 59 | 62 | 50 | 55 |
| | 9 | 10 | 55 | 58 | 47 | 50 |
| | 20 | 9 | 52 | 50 | 49 | 54 |
| | 18 | 20 | 60 | 64 | 50 | 52 |
| | 14 | 19 | 58 | 61 | 68 | 65 |
| | 10 | 16 | 65 | 68 | 58 | 60 |
| Mean | 12.2 | 12.5 | 56.7 | 59.1 | 51.5 | 54.5 |

*Table II* Percentage of T lymphocytes

| | Peripheral blood | Tonsil | Appendix |
|---|---|---|---|
| | 45 | 28 | 27 |
| | 60 | 26 | 31 |
| | 54 | 28 | 25 |
| | 50 | 23 | 25 |
| | 52 | 20 | 24 |
| | 48 | 23 | 37 |
| | 52 | 27 | 34 |
| | 47 | 22 | 35 |
| | 46 | 21 | 18 |
| | 46 | 18 | 23 |
| Mean | 50 | 23.6 | 27.9 |

planations may be offered for the present findings (1) the presence of chronic inflammation may be the cause of regional accumulation of B lymphocytes although in two appendices where no inflammation existed the percentage of B cells was equally high, or (2) the high percentage of B lymphocytes found in tonsils and appendices could support the hypothesis that the lymphoid tissue associated with the gastrointestinal system may constitute the equivalent of the bursa in mammals

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# Activity of Inhibitors on Plasmatic Erythropoietin and on the Renal and Splenic Erythropoietic Factors in the Rabbit

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*Key Words* Cleland's reagent Erythropoiesis Erythropoietin Koshland's reagent Renal erythropoietic factor Splenic erythropoietic factor

*Abstract* The author has studied the action of Cleland's reagent and Koshland's reagent on the erythropoietic factors present in the kidneys and the spleen of normal rabbits, in the plasma of bled rabbits and in the mixtures obtained through incubation of normal plasma with kidney and spleen homogenates. The results show that Cleland's reagent inhibits the activity of the renal and splenic erythro-stimulant factors, whereas it has no effect upon the erythropoietin in anaemic plasma and in plasma kidney and plasma spleen incubation mixtures. The activity of erythropoietin, on the other hand, is inhibited by Koshland's reagent, which has no influence on the renal and splenic erythro-stimulant factors.

LEWIS *et al.* [5] have identified the presence of erythro-stimulant factors in the urine of anaemic patients, in particular, in addition to erythropoietin, an erythropoietic factor has also been found, possessing certain characteristics similar to those of the renal factor studied by CONTRERA and GORDON [1] and by ZANJANI *et al.* [13], since it gives rise to the production of erythropoietin if incubated with serum of normal rabbits.

LEWIS *et al.* [6] later studied the effects of Cleland's reagent (2,3-dihydroxy 1-4-dithiolbutane or dithiothreitol, DTT), known for its capacity to hinder the oxidation of the –SH groups, on the two different erythro-stimulant factors in urine. They showed that DTT was capable of completely inactivating the erythropoietic factor content in urine, whereas it had no effect on the erythropoietin, even in very high doses. It therefore seems that the activity of the erythropoietic factor is connected with the presence of –SH groups, though these are not necessary for the bio-

logical activity of erythropoietin, as is also shown by the studies of GOLDWASSER and KUNG [4]. Moreover, also LOWY and KEIGHLEY [11] state specifically that DTT does not inactivate erythropoietin.

*On the other hand, these last authors have shown [11] that erythropoietin is inactivated by* Koshland's reagent (2-hydroxy-5-nitrobenzylbromide), which reacts with the tryptophane in the protein chains, forming substitution products with the indolic ring and leaving the peptide bonds unharmed.

The object of the present studies was to verify whether DTT, known for its capacity to inhibit the erythropoietinogenic factor present in the urine of anaemic patients [6], was equally capable of inactivating the renal erythropoietic factor and the erythropoietic factor present in the spleen of the rabbit, which it also considered to be of renal origin [8]. Another object of the studies was to verify whether Koshland's reagent, known for its capacity to inhibit erythropoietin, was equally capable of inhibiting the activity of the plasma-kidney and plasma-spleen incubation products, which probably contain erythropoietin [9]. A final object was to establish if the systems considered responded differently to the same inhibitor, in order to derive further confirmation of the hypothesis of the different nature of the factors contained in the kidney and in the spleen, compared with those contained in anaemic plasma or in plasma-kidney and plasma-spleen incubation products.

## *Material and Methods*

*Extraction of the renal and splenic factors* The light mitochondrial and microsomal fractions of the kidney and the mitochondrial fraction of the spleen, containing the erythro-stimulant factors were isolated from normal rabbits [3] The hypotonic extracts of these subcellular fractions were then subjected to fractionation with ammonium sulphate in concentrations of 30 and 70% saturation, in accordance with the first stage of the technique followed by WONG *et al* [12] The two fractions thus obtained were suspended again in 20 ml of bi distilled water and subjected to dialysis in order to eliminate the ammonium sulphate On evaporating, in vacuum, the dialysed product relative to the fractions obtained from the kidneys or from the spleen with the second precipitation – carried out, as has been stated, with ammonium sulphate at 70% saturation – a residual material was obtained, weighing on the average, 48 mg (kidney) and 9 8 mg (spleen), with very small variations in these values

*Extraction of the plasmatic factors* With similar aims, an attempt was made to obtain, in sufficiently concentrated form, both the erythropoietin contained in the plasma from bled rabbits (20 ml/kg of body weight), 24 h after bleeding and that

present in the incubation mixtures prepared with plasma and kidney or spleen homogenates from normal rabbits [9]. For these experiments a technique similar to that described by LOWY and BORSOOK [10] was followed; in particular, both the anaemic plasma relative to each donor (50 ml) and the above-mentioned incubation products (50 ml of normal plasma mixed with the hypotonic homogenate of a kidney or a spleen) were heated in boiling water and then cooled and centrifuged. In order to obtain a more effective precipitation of the thermo-labile protein substances present in the materials studied 1-1.5 g of NaCl was added before heating since the acidification of the material carried out according to LOWY and BORSOOK's [10] technique might have given rise in the presence of spleen homogenate, to the inactivation of the erythropoietin in the incubation mixtures obtained with that organ. In the course of previous studies [9] the splenic tissue was found to possess a factor capable of inactivating or degrading erythropoietin in an acid environment. The supernatant obtained by the above mentioned methods was then subjected to fractionation by means of absolute ethanol and the fraction precipitated with 60-80% concentrations of ethanol was then dried in vacuum and weighed. The average weight of these fractions was 21.2 mg for the anaemic plasma, 23.5 mg for the plasma spleen incubation material and 30.1 mg for the plasma kidney incubation material.

The active fractions of renal, splenic or plasmatic origin obtained by the methods described above were then subjected to the action of certain inactivating substances (DTT and Koshland's reagent) in order to verify whether they were capable of suppressing the erythro-stimulant activity. These experiments were performed in conjunction with others as a control in order to verify the existence of an activity in the preparations used.

*Cleland's reagent and renal splenic erythropoietic factors.* The active material corresponding to the kidneys of a donor and prepared by the methods described above was incubated for 30 min with 5 mg of DTT at 37 °C [6]. Preliminary experiments carried out by using scalar doses of this reagent had shown that a dose of 5 mg was sufficient to inactivate the erythropoietic factor contained in the kidneys of a normal animal. Similarly, the active fraction corresponding to a spleen was treated with 1 mg of DTT – the dose calculated in relation to the average weight of the residual material obtained by evaporating the fraction in vacuum (9.8 mg), which is found to be about one fifth of that of the material obtained with the same methods from kidney extracts (45 mg).

*Koshland's reagent and plasmatic erythro-stimulant factors.* The preparations rich in erythropoietin obtained from the anaemic plasma or from the kidney plasma and spleen plasma incubation mixtures by the methods already described, were dried in vacuum and were then dissolved in bi-distilled water in a concentration of 0.1%. To these preparations an equal quantity of Koshland's reagent dissolved in 0.5 ml of acetone was added. The reaction mixture was then kept at 23 °C for 1 h and subjected to dialysis against bi-distilled water in order to eliminate the acetone and the reagent [11].

*The reverse use of the reagents on the erythropoietic factors.* In the course of preliminary tests, Koshland's reagent was found to have absolutely no effect on the renal and splenic erythropoietic factors, when added to the active fractions in a quantity equal to their average weight. Other tests were therefore carried out using this reagent in very high doses, equal to 10 times the average weight of the fractions

(480 mg in the inactivation tests carried out on the renal material and 98 mg in those carried out on the splenic material) These doses were dissolved in 10 ml and in 2 ml of acetone, respectively, and then added to the renal or splenic material under examination The incubation and the subsequent dialysis were performed with the usual technique

Similar inactivation tests were then carried out by incubating DTT, known for its inability to inhibit erythropoietin [6, 11] with active fractions prepared from kidney plasma and spleen plasma mixtures, also making use of fractions containing erythropoietin, since they were obtained from anaemic plasma Also in these experiments, high doses of the reagent were used (20–30 mg of DTT), being proportionally equal to 10 times those sufficient to inhibit renal or splenic active fractions having the same weight, since preliminary tests, carried out by using such doses (2–3 mg of DTT) had given negative results

With the preparations thus obtained, various experiments were performed First of all, the erythro-stimulant activity of the various extraction materials was studied. A test was then made to verify whether DTT and Koshland s reagent were capable of suppressing the erythro stimulant activity present in these materials.

These activities were assessed by studying the variations in the reticulocyte pattern in the peripheral blood of rigidly selected rabbits, later treated with the various preparations under examination by following the criteria already described in previous works [2, 3] In particular, in the extraction tests for the various erythro-stimulant factors, use was made of groups of 6 normal or bled donors, from which organs or plasma were removed and, at the same time, of groups of 6 receivers, each of which was to be treated with the material obtained from a single donor On the other hand, in the inactivation tests including also the control experiments, use was made of groups of 12 donors, killed two at a time The active fractions obtained from each pair of animals were combined into a single complex, half of which was incubated with the inactivator under study and the remaining half used in the control experiment Also in these tests, groups of 6 receivers for each preparation to be tested were used

The material to be administered to each animal was in every case, brought up to a volume of 40 ml by means of bi distilled water, made isotonic by adding NaCl, divided into two doses to be given by intraperitoneal injection at an interval of 24 h and kept at –20 °C until use

## *Results*

The results obtained by the treatment with fractions of subcellular extracts of kidney and of spleen (table I) show that in both organs the fractions sedimented with ammonium sulphate at 30–70% saturation (groups 1B, D) produce a highly significant increase in the reticulocyte content whereas those sedimented with 30% ammonium sulphate (groups 1A, C do not produce any significant variation in the number of circulating reticulocytes These active fractions of subcellular extracts of kidney and of

*ble I* Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented usi $(NH_4)_2SO_4$ at 30% (groups 1A, C) and at 30–70% (groups 1B, D) saturation from a hypotonic extract light mitochondrial and microsomal fractions of kidney (groups 1A, B) or of the mitochondrial fractio of the spleen (groups 1C, D) of normal donors

| | Group 1A | | Group 1B | | Group 1C | | Group 1D | |
|---|---|---|---|---|---|---|---|---|
| | mean ± SE | t test | mean ± SE | t-test | mean ± SE | t test | mean ± SE | t tes |
| itial | 2.3 ±0 0931 | | 2.35±0 0957 | | 2.6 ±0 1291 | | 2.35±0 1310 | |
| lays | 2.55±0 0957 | 1 872 | 3 55±0 1522 | 6 674[2] | 2.75±0 1310 | 0 8157 | 3 97±0.1406 | 8 43 |
| lays | 2 15±0 1384 | 0 8992 | 3 95±0 1384 | 9 423[2] | 2.7 ±0 1065 | 0 5976 | 4 35±0 1747 | 9 16 |
| lays | 2.5 ±0 1506 | 1 13 | 3 45±0 1648 | 5 771[2] | 2.82±0 1514 | 1 105 | 3 82±0 1301 | 7 96 |
| lays | 2.65±0 1668 | 1 832 | 2.55±0 1176 | 1 322 | 2.62±0 1352 | 0 107 | 2.58±0 1492 | 1 1[illegible] |

[1] The reticulocyte level is expressed as a reticulocyte/erythrocyte percentage. Mean values ± standa ror and comparison between the averages of the initial and subsequent values by means of Student's t-t l f 10)

[2] Highly significant.

*able II* Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented usi $(NH_4)_2SO_4$ at 30–70% saturation from a hypotonic extract of the light mitochondrial and microson ractions of kidney (groups 2A, B) or of the mitochondrial fraction of the spleen (groups 2C, D) of norm donor, given as a control (groups 2A C) or after further incubation with DTT (groups 2B, D)

| | Group 2A (control) | | Group 2B | | Group 2C (control) | | Group 2D | |
|---|---|---|---|---|---|---|---|---|
| | mean ± SE | t-test | mean ± SE | t test | mean ± SE | t test | mean ± SE | t te |
| nitial | 2.15±0 1118 | | 2.32±0 1352 | | 2.42±0 1077 | | 2.52±0 1249 | |
| days | 3 75±0 1204 | 9 737[2] | 2.57±0 1115 | 1 427 | 3 78±0 1301 | 8 052[2] | 2.7 ±0 1592 | 0 6[illegible] |
| days | 4 0 ±0 1732 | 8 974[2] | 2.3 ±0 1633 | 0 0943 | 4 15±0 1232 | 10 57[2] | 2.63±0.1256 | 0 6 |
| days | 3.58±0 1137 | 8 967[2] | 2.2 ±0 1155 | 0 6751 | 3 82±0 1492 | 7 607[2] | 2.62±0 1424 | 0 5 |
| 5 days | 2.33±0 0985 | 1.206 | 2.25±0 1354 | 0.3618 | 2.65±0 1384 | 1.311 | 2.7 ±0 1317 | 0 9[illegible] |

[1, 2] See table II

spleen, if previously incubated with DTT (table II), lose their erythro-stimulant capacity, since – in consequence of this incubation – they are no longer able to produce any significant variation in the reticulocyte pattern in the treated animals (groups 2B, D)

If the same active fractions are incubated with Koshland's reagent

*Table III* Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented w[ith] $(NH_4)_2SO_4$ at 30–70% saturation from a hypotonic extract of the light mitochondrial and microsomal fractions of kidney (groups 3A, B) or of the mitochondrial fraction of the spleen (groups 3C, D) of normal donor, given as a control (groups 3A, C) or after further incubation with Koshland's reagent (groups 3B, D)

| | Group 3A (control) | | Group 3B | | Group 3C (control) | | Group 3D | |
|---|---|---|---|---|---|---|---|---|
| | mean±SE | t test | mean±SE | t test | mean±SE | t test | mean±SE | t test |
| Initial | 2 68±0 1621 | | 2.2 ±0 1238 | | 2 45±0 1204 | | 2 12±0 1400 | |
| 2 days | 3 85±0 1432 | 5 41[2] | 3 65±0 1628 | 7 09[2] | 3 6 ±0 1483 | 6 02[2] | 3 62±0 1740 | 6.71[2] |
| 3 days | 4 28±0 1579 | 7 07[2] | 3 92±0 1579 | 8 57[2] | 3 88±0 1579 | 7 202[2] | 3 75±0 1455 | 8 07[2] |
| 4 days | 4 0 ±0 1592 | 5 811[2] | 3 72±0 1778 | 7 016[2] | 3 63±0 1308 | 6 638[2] | 3 48±0 1166 | 7 46[2] |
| 6 days | 2 95±0 1544 | 1 206 | 2 37±0 1406 | 0 9074 | 2 32±0 1275 | 0 7411 | 2 38±0 1352 | 1 38 |

[1] [2] *See table I*

*Table IV* Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating, centrifugation and fractionation with $C_2H_5OH$ at 60–80% concentration from plasma of donor bled 24 h before (group 4A) or from a kidney-plasma (group 4B) or spleen plasma (group 4C) incubation mixture of normal donors

| | *Group 4A* | | *Group 4B* | | *Group 4C* | |
|---|---|---|---|---|---|---|
| | mean ± SE | t test | mean ± SE | t test | mean ± SE | t test |
| Initial | 2 63±0 1030 | | 2 62±0 1424 | | 2 32±0 1166 | |
| 2 days | 3 88±0 1400 | 7 136[2] | 3 92±0 1641 | 5 948[2] | 3 55±0 1522 | 6 415[2] |
| 3 days | 4 33±0 1801 | 8 148[2] | 4 33±0 1453 | 8 407[2] | 3 92±0 1470 | 8 525[2] |
| 4 days | *3 72±0 1661* | *5 541[2]* | *3 75±0 1708* | *5 083[2]* | *3 67±0 1382* | 7 46[2] |
| 6 days | 2 92±0 1887 | 1 342 | 2 57±0 1605 | 0 2331 | 2 45±0 1310 | 0 7411 |

[1] [2] See table I

however, even if it is used in strong doses (table III), they preserve their erythro-stimulant capacity Even after this incubation, they produce a highly significant increase in the reticulocyte pattern (group 3B, D)

The material obtained by means of heating, centrifuging and fractionation with 60–80% ethanol (table IV), whether it comes from plasma of an anaemic donor (group 4A) or from incubation mixtures kidney-normal plasma (group 4B) or spleen-normal plasma (group 4C) produces a highly significant increase of reticulocytes However, in comparison with the two

*Table V* Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating, centrifugation and fractionation with $C_2H_5OH$ at 60-80%, concentration from plasma of a donor bled 24 h before (groups A, a) and from kidney-plasma (groups B, b) or spleen plasma (groups C, c) incubation mixture relative to a normal donor, given as a control (groups A, B, C) or after further incubation with DTT (groups a, b, c)

| | Group 5A (control) | | Group 5a | |
|---|---|---|---|---|
| | mean ± SE | t test | mean ± SE | t test |
| Initial | 2.5 ± 0.1315 | | 2.27 ± 0.1054 | |
| 2 days | 3.82 ± 0.1470 | 6.69[2] | 4.02 ± 0.1424 | 9.881[2] |
| 3 days | 4.28 ± 0.1447 | 9.099[2] | 4.15 ± 0.1204 | 11.75[2] |
| 4 days | 3.63 ± 0.1229 | 6.275[2] | 3.72 ± 0.1194 | 9.104[2] |
| 6 days | 2.75 ± 0.1335 | 1.333 | 2.48 ± 0.1470 | 1.161 |
| | **Group 5B (control)** | | **Group 5b** | |
| | mean ± SE | t test | mean ± SE | t test |
| Initial | 2.12 ± 0.1077 | | 2.38 ± 0.1272 | |
| 2 days | 3.68 ± 0.1579 | 8.162[2] | 3.57 ± 0.1475 | 6.049[2] |
| 3 days | 3.93 ± 0.1282 | 10.81[2] | 3.82 ± 0.1711 | 6.7[2] |
| 4 days | 3.58 ± 0.1514 | 7.857[2] | 3.65 ± 0.1455 | 6.506[2] |
| 6 days | 2.12 ± 0.1166 | 0 | 2.27 ± 0.1202 | 0.6211 |
| | **Group 5C (control)** | | **Group 5c** | |
| | mean ± SE | t test | mean ± SE | t-test |
| Initial | 2.18 ± 0.1352 | | 2.58 ± 0.1249 | |
| 2 days | 3.65 ± 0.1500 | 6.28[2] | 3.75 ± 0.1565 | 5.843[2] |
| 3 days | 3.85 ± 0.1658 | 7.806[2] | 4.22 ± 0.1923 | 7.156[2] |
| 4 days | 3.72 ± 0.1275 | 8.287[2] | 3.57 ± 0.1144 | 5.844[2] |
| 6 days | 2.33 ± 0.1201 | 0.8295 | 2.75 ± 0.1360 | 0.9206 |

[1, 2] See table I

reagents previously employed, these fractions display an opposite behaviour. If these active fractions are incubated with DTT (table V) before being administered to receiver animals, they preserve their erythro-stimulant capacity (groups 5a–c), since they produce highly significant variations in the reticulocyte pattern, which are exactly like those found in control animals (groups 5A–C). This erythro-stimulant activity persists if

*Table VI* Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating centrifugation and fractionation with $C_2H_5OH$ at 60–80°, concentration from plasma of a donor bled 24 h before (groups A, a) and from a kidney plasma (groups B, b) or spleen plasma (groups C, c) incubation mixture relative to a normal donor, given as a control (groups A–C) or after further incubation with Koshland's reagent (groups a–c)

| | Group 6A (control) | | Group 6a | |
|---|---|---|---|---|
| | mean ± SE | t test | mean ± SE | t test |
| Initial | 2.05 ± 0.1232 | | 2.15 ± 0.1283 | |
| 2 days | 3.57 ± 0.1406 | 8.133[2] | 2.0 ± 0.1211 | 0.8496 |
| 3 days | 3.82 ± 0.1352 | 9.68[2] | 2.32 ± 0.1327 | 0.9206 |
| 4 days | 3.38 ± 0.1600 | 6.857[2] | 2.27 ± 0.0954 | 0.75 |
| 6 days | 2.27 ± 0.1453 | 1.155 | 2.28 ± 0.1249 | 0.7256 |
| | **Group 6B (control)** | | **Group 6b** | |
| | mean ± SE | t test | mean ± SE | t test |
| Initial | 2.45 ± 0.1148 | | 2.7 ± 0.1366 | |
| 2 days | 3.67 ± 0.143 | 6.656[2] | 2.92 ± 0.1778 | 0.9812 |
| 3 days | 4.13 ± 0.152 | 8.821[2] | 2.92 ± 0.2056 | 0.8913 |
| 4 days | 3.5 ± 0.1265 | 6.148[2] | 2.73 ± 0.1308 | 0.1586 |
| 6 days | 2.65 ± 0.1384 | 1.112 | 2.85 ± 0.1586 | 0.7163 |
| | **Group 6C (control)** | | **Group 6c** | |
| | mean ± SE | t test | mean ± SE | t test |
| Initial | 2.33 ± 0.1256 | | 2.72 ± 0.1514 | |
| 2 days | 3.35 ± 0.1408 | 5.936[2] | 2.53 ± 0.152 | 0.8856 |
| 3 days | 3.82 ± 0.1376 | 8.536[2] | 2.63 ± 0.1453 | 0.4289 |
| 4 days | 3.27 ± 0.1308 | 5.736[2] | 2.6 ± 0.1183 | 0.6244 |
| 6 days | 2.55 ± 0.1384 | 1.712 | 2.87 ± 0.1333 | 0.7435 |

[1] [2] See table I

the DTT is used in much higher doses than those sufficient to inhibit the preparations obtained from the kidney and from the spleen

The fractions obtained from the anaemic plasma and from the kidney-plasma and spleen plasma incubation mixtures lose their erythro stimulant capacity if they are incubated with Koshland's reagent before being used (table VI) In fact, in these experimental conditions they do not produce any significant variation in the reticulocyte pattern (groups 6a–c)

*Discussion*

Assessing the results obtained by means of treatment with protein fractions of renal or splenic subcellular extracts, it may be stated that, if the erythro-stimulant efficacy of the treatment carried out with the fraction of renal extract sedimented with ammonium sulphate at 30–70% saturation could also have been taken for granted to a certain extent – in consideration of the results already obtained by Wong *et al* [12] in the rat – the similar activity present in the splenic fraction obtained with the same technique nevertheless constitutes a new element, since the role of the spleen in the erythropoiesis of the rabbit has been explained only by the studies carried out in this Institute in recent years [7, 8]

On the basis of the results obtained by administering renal or splenic fractions rich in erythropoietic factors, after incubation with DTT, it appears that this reagent, in the experimental conditions adopted, inactivates the factor previously identified in the renal tissue of the rabbit [3, 8] It is therefore probable that it is to be identified with that described by Lewis *et al* [5, 6] in the urine of anaemic patients a hypothesis also suggested by the results of other recent studies [2] The fact that in the same experimental conditions the DTT also inactivates the factor present in the spleen of the rabbit appears to be in agreement with the results of previous works, which show that there occurs, in the spleen, an accumulation of an erythropoietic factor produced and released into the circulation by the kidney [7, 8]

The hypothesis that the erythropoietic factors contained in the kidney and in the spleen are of the same nature is also strengthened by the fact that the erythro stimulant activity of both is inhibited by the DTT but not by Koshland s reagent These factors cannot be identified with erythropoietin, since this is not inactivated by the DTT [6, 11] whereas Koshland s reagent is an effective inhibitor of it [11] Moreover, the fact that the active fractions obtained from kidney plasma and spleen plasma incubation mixtures, like those obtained from anaemic plasma, do not lose their erythro-stimulant activity if treated with DTT, whereas they lose it completely if treated with Koshland s reagent, shows that these mixtures, like anaemic plasma do not contain the same erythropoietic factors as those present in the kidney and in the spleen, but contain erythropoietin

All these data therefore show that the erythropoietic factors present in the kidney and in the spleen differ from the erythropo etin to be found in anaemic plasma and in kidney normal plasma and spleen normal plasma

incubation mixtures, not only in their molecular weight, as recently demonstrated [2], but also in other physicochemical characteristics

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# Cephalothin-Induced Immune Hemolytic Anemia

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*Key words* Cephalothin Drug induced hemolytic anemia Hemolytic anemia Immune reactions

*Abstract* A patient with renal disease developed Coombs positive hemolytic anemia while receiving cephalothin therapy An anti-cephalothin IgG antibody was detected in the patient's serum and in the eluates from her erythrocytes. In addition, nonimmunologic binding of normal and patient's serum proteins to her own and cephalothin-coated normal red cells was demonstrated Skin tests and *in vitro* lymphocyte stimulation revealed that the patient was sensitized to cephalothin and also to ampicillin Careful investigation of drug induced hemolytic anemias reveals the complexity of the immune mechanisms involved

Drug-induced immune hemolytic anemias are uncommun despite the fact that many drugs lead to the development of antibodies However, some of these drugs, such as penicillin, bind firmly to red cells *in vitro* and more than 20 cases of penicillin-induced hemolytic anemia have been reported [11] Cephalothin and other cephalosporins which are chemically related to penicillin, also bind to red cells *in vitro* Cephalothin sodium (Keflin) has been associated with a positive direct Coombs test in many patients receiving this antibiotic [4, 7] but only 2 cases of cephalothin and 1 case of cephalexin-induced immune hemolytic anemia have been reported as yet [2, 5] The purpose of this paper is to report an additional case of Coombs-positive hemolytic anemia related to cephalothin therapy and to define the characteristics of immunohematologic reactions to this drug

*Table II* Coombs test after incubation of erythrocytes with cephalothin or penicillin using patient's serum, patient's erythrocyte eluate or normal serum

| | Cephalothin-coated erythrocytes | | | Penicillin-coated erythrocytes | | | Normal erythrocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | *broad-spectrum* | *IgG* | *C3* | *broad-spectrum* | *IgG* | *C3* | *broad spectrum* | *IgG* | *C3* |
| *Patient's serum* | 1 512 | 1 512 | 1 32 | 1 2 | ND | ND | – | – | – |
| Patient's eluate | 1 4 | ND | ND | – | ND | ND | – | ND | ND |
| Normal serum 1 | 1 256 | 1 256 | 1 32 | 1 4 | ND | ND | – | ND | ND |
| Normal serum 2 | 1 128 | 1 256 | 1 32 | – | ND | ND | – | ND | ND |

ND = Not done

patient's erythrocytes agglutinated cephalothin-coated erythrocytes, but did not agglutinate penicillin-coated erythrocytes or normal erythrocytes That the antibody in patient's serum and eluate belonged to the IgG class was confirmed by the absence of modification of the agglutination titer of cephalothin-coated erythrocytes following 2-mercaptoethanol treatment Two *normal sera from blood donors were used as controls* Both were found to agglutinate cephalothin-coated erythrocytes almost as strongly as the patient's serum using the same broad-spectrum, anti-IgG and anti-C3 antisera (table II)

*Fractionation studies* 10/ml of the patient's serum obtained 15 days following interruption of therapy and when direct Coombs test was strongly positive were chromatographed on Sephadex G-200 The elution diagram was arbitrarily divided into 5 fractions which were concentrated to obtain twice the original volume of serum These fractions were assayed with cephalotin-coated erythrocytes and antisera to IgG, IgA, IgM, C3, *transferrin and β-lipoprotein* Fractions III and IV which disclosed strong agglutination with anti-IgG, anti-IgA and anti-transferrin were pooled and recycled on Sephadex G-200 Three fractions were obtained which were analyzed by immunoelectrophoresis and by radial diffusion in agar (Tri-Partigen, Behringwerke, Marburg/Lahn, FRG) to evaluate their content in immunoglobulins, C3 and transferrin These fractions were again assayed with cephalothin-coated erythrocytes and antisera The same procedure was performed with a pool of two normal blood donors

*III* Serum proteins present in fractions from patient's and normal sera following fractio Sephadex G 200

| | Radial immunodiffusion | | | | | Immunoelectrophoresis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | total | IgG | IgA | IgM mg ml | trans-ferrin | IgG | IgA | IgM | trans-ferrin | β-lipo prote |
| *tions from patient's serum* | | | | | | | | | | |
| | ND | 4.0 | 0.34 | 0.08 | 0 | + | (+) | – | – | – |
| | ND | 17.0 | 2.68 | 0 | 0 | + + + | + + + | – | – | – |
| | ND | 27.7 | 0.66 | 0 | 0 | + + + + | + | – | – | – |
| *tions from a pool of two normal sera* | | | | | | | | | | |
| | 3.0 | | | | | – | – | – | – | – |
| | 27.0 | | ND | | | + + | + | – | + | – |
| | 18.0 | | | | | + + | + + | – | – | – |

*le II* Coombs tests after incubation of cephalothin-coated erythrocytes[1] using fractions fro and normal sera

| sera[2] | Broad spectrum | IgG | IgM | IgA | Trans-ferrin | β-lipo-protein |
|---|---|---|---|---|---|---|
| *ctions from patient's serum* | + + + | + + + | – | + + + | + + + | + + |
| | + | + | – | + + + | + + | + |
| | + | + | – | + + + | + | + |
| | + | + + + | – | + + | – | – |
| *ctions from normal sera* | + + + | + + + | – | + + + | + + + | + + |
| | – | + | – | + | – | – |
| | + | + | – | + | – | – |
| | + | + + | – | – | – | – |

[1] Non-coated erythrocytes were negative with all antisera

[2] All antisera used at dilution 1 8

Table III shows the concentrations of the different serum proteins found in the three fractions used IgG was recovered mainly in fractions II and III IgA was found in all fractions but especially in fraction II When incubated with the three fractions, cephalothin-coated erythrocytes were found to absorb serum proteins from each fraction in a nonspecific

*Table V.* Patient's lymphocyte stimulation in culture using penicillin, ampicillin and cephalothin as antigen performed on 2 different days (mean of 2 tests)

| | 16.8.70 cpm | 18.9.70 cpm |
|---|---|---|
| Lymphocytes alone | 513 | 472 |
| Lymphocytes + penicillin G | 971 | 728 |
| Lymphocytes + ampicillin | 1,119 | 884 |
| Lymphocytes + cephalothin | 1,096 | 1,216 |
| Lymphocytes + PPD (10 μ) | 1,582 | 1,390 |
| Lymphocytes + phytohemagglutinin | 63,620 | 54,620 |

way. Moreover (table IV), no clear-cut difference could be seen between the fractions from the patient's serum and those from normal serum. The serum proteins most readily absorbed by this apparently nonimmunologic mechanism were IgG, IgA, transferrin and β-lipoprotein.

*Evidence for sensitization to cephalothin.* Skin tests were performed in the patient, 9 days after therapy with cephalothin had been interrupted. A positive skin test was observed after intradermal injection of penicilloyl-polylysine, 250 mmol/ml), the last test was stronger than the others. Lymphocyte culture tests were also performed on two different days, using the same three drugs as antigens (table V). All three tests induced moderately positive lymphocyte stimulation.

## *Discussion*

The reported incidence of positive Coombs test in patients receiving cephalothin has varied between 3.4 and 75% [4, 7, 9, 11]. These reactions were often weak and occurred from one to a few days after starting treatment. They were not associated with hemolysis. In some studies, the dosage and duration of therapy were found to influence the incidence of positive reactions. A large percentage of patients with positive Coombs tests had renal disease with high blood levels of cephalothin secondary to reduced renal excretion. As suggested by GRALNICK *et al.* [4], hypoalbuminemia may also favor the occurrence of positive Coombs tests, the albumin fraction being responsible for over 95% of the serum binding of cephalothin [6].

Previously proposed mechanisms regarding the pathogenesis of posi-

tive Coombs test without hemolysis, occurring in patients receiving cephalothin include (1) a nonimmunologic binding of serum proteins to red cells [4, 7, 11] and (2) an immune reaction involving an antibody cross-reacting with cephalothin- or penicillin-coated red cells [4, 8, 11]. As indicated by SPATH *et al.* [11], both mechanisms seem to occur in parallel in these patients. Nonimmunological absorption of proteins, especially albumin, to cephalothin-coated red cells could be demonstrated *in vitro*, multiple absorptions of a serum with cephalothin-sensitized erythrocytes revealed that these cells absorb IgG and other proteins, including complement (C3) and albumin [11]. Our own data confirm this nonimmunologic binding of proteins and revealed that IgA, transferrin and $\beta$ lipoprotein may also be absorbed from normal sera by cephalothin-coated erythrocytes (table III). In addition SPATH *et al.* [11] have demonstrated that an immune mechanism may also cause positive direct antiglobulin tests *in vivo* during cephalothin therapy. Eluates from the patient's erythrocytes react with cephalotin and penicillin sensitized erythrocytes. This was not a nonspecific absorption of protein because eluates from red cells sensitized with an anti Rh IgG isoantibody would not bind IgG to cephalothin-coated red cells lacking the appropriate antigen. In addition, SPATH *et al.* [11] showed that the sera of 4 patients having a positive antiglobulin test all contained high titers of IgG cephalothin antibodies, whereas in the other treated patients IgM antibodies were found in 88% and IgG antibodies in only 25% of cases.

Despite the frequent occurrence of a positive direct Coombs test in patients receiving cephalothin only 2 cases of hemolytic anemia associated with cephalothin have been described so far [5]. As in our case, erythrocytes from these patients were strongly positive in the direct Coombs test with broad spectrum anti human globulin reagents and with a specific anti IgG antiserum. Our patient's erythrocytes were also strongly agglutinated by an anti-complement (C3) reagent. Weak reactions were also obtained with anti IgM, anti IgA and anti transferrin reagents, suggesting the simultaneous occurrence of nonimmunological binding of serum proteins. In our case as in the others, a specific IgG cephalothin antibody was demonstrated in eluates from patients' erythrocytes. However, as mentioned above this specific immune reaction was also observed in patients with positive direct Coombs test without hemolysis and therefore cannot be used as a definitive proof that cephalothin caused the hemolytic anemia. Presumably, as suggested by WORLLEDGE [12] the quality and quantity of the antibody are also important.

# ANNOUNCEMENT

## Indian Council of Medical Research

### Colonel Amir Chand Trust Prizes for Medical Research

t-Colonel Amir Chand lately Principal of Lady Hardinge Medical ge New Delhi has donated Rs 50 000/ to the Indian Council of al Research for creating a prize fund From the interest earned by n prizes are to be awarded for the best published research work in medical rs The Governing Body of the Council has constituted a Trust called Colonel Amir Chand Trust for the administration and management of the

The prizes are awarded annually on an All India basis for the best published rch work in any subject in the field of medical sciences including clinical arch The term Clinical Research covers research into the mechanism causation of disease and its prevention and cure and includes work on in hospitals field studies in epidemiology and social medicine and rvations in general practice

It has been decided to award *four prizes* in 1959 each of Rs 300/ to s of not more than ten years standing for the best research papers in Th zes will be known

*GRADUATES*

Selection Board

In the case of joint authorship of a publication the prize shall be divided en the authors in such proportion as the Selection Board may decide r of the person who applies for the prize should be clearly indicated to make it easy to determine whether the *major part* of the work has done by that person

THE AWARD of the prizes will be announced at the annual meetings of Council's Scientific Advisory Board and Advisory Committees in December 1959

CANDIDATES for award of prizes are required to submit 10 REPRINTS of r papers published during 1958 These should be sent to the DIRECTOR IAN COUNCIL OF MEDICAL RESEARCH P O BOX 494 NEW DELHI so as to reach NOT LATER THAN 1ST AUGUST 1959 THE PAPERS should be accompanied a short biographical sketch and two copies of passport size photographs of candidate/candidates concerned

# NEWS & NOTES

In the April 1958 issue of our journal on page 143 the following news item was published under the heading "Treatment of leprosy patients under the Ayurvedic System". In this connection Dr Wardekar the Secretary of the Gandhi Memorial Leprosy Foundation Wardha has very kindly sent us the following information:—

The Gandhi Memorial Leprosy Foundation (Leprosy section of the Gandhi Smarak Nidhi) does not have any proposal to start a 200 bedded hospital for routine Ayurvedic Treatment. It has decided only to undertake for a period of 2 years a trial of Ayurvedic drugs with the sole object of studying the efficacy of these drugs on a few selected cases of leprosy. Arrangements have been made to accommodate only 10 male and 10 female patients. The trial will be conducted by a Registered body to be formed by Shri Parthanarayana Pandit for this purpose.

open A separate consideration is perhaps indicated for the combined deficiency of factors VII and X together with carotid body tumors described in several members of a large kindred [20] The cases of hemophilia B with associated factor VII deficiency are also doubtful since they could represent cases of hemophilia $B_M$ [15, 28]

Combined deficiency of factors VII and VIII has been satisfactorily documented only in 1 patient [5] and suspected in 3 additional cases [2, 19] In only 1 instance the defect has been demonstrated in family members of the propositus [19] Furthermore, even in the case shown to have low factors VIII and VII by specific assay [5], the possibility of a non hemophilic factor VIII defect was not ruled out [5]

The object of the present study is to report a patient who presented a mild hemophilia A and moderate factor VII deficiency The same defect was present in several family members of both sexes Therefore, this seems to be the first family presenting a combined defect of factors VII and VIII

## *Case Report*

The propositus is a 22 year old male who was first seen by us in Padua on December 17, 1974 and had been followed by one of us (R V) for the past 4 years The family history was partially positive for bleeding tendency (fig 1) The mother presented easy bruising menomethrorrhagia and *post partum* bleeding A maternal aunt presented menomethrorrhagia A maternal cousin had bled excessively after brain surgery for a benign tumor and easy bruising Another maternal cousin was known to be prone to gingival bleeding The parents were not consanguineous and came from different areas

At the age of 6 the patient bled profusely after tonsillectomy Bleeding stopped after two local packings and a fresh blood transfusion The blood transfusion, however, had to be interrupted because of a reaction At the age of 7 the patient was involved in a car accident and sustained a fracture of the left femur and a laceration of the upper lip Bleeding from the wound was marked He was admitted to a hospital in Turin where he was sutured and a cast was applied The healing of the fracture was very slow since it required a 3 month period before the cast could be removed At the age of 8 the patient had an abdominal trauma which caused melena Bleeding subsided after a few days of bed rest At the age of 10 the patient presented excessive bleeding after a deciduous tooth extraction At that time he was suspected to have hemophilia A

At the age of 18 the patient presented severe bleeding after a molar tooth extraction He had to be admitted to a local hospital and bleeding subsided only after transfusion of fresh frozen plasma During this admission, a routine coagulation study revealed a moderate factor VII defect together with a mild prolongation of the partial thromboplastin time This latter finding remained unexplained

Easy bruising and epistaxes have been present since childhood Hematuria and hemarthrosis have never occurred At the time of study there were no bleeding manifestations

Physical examination was negative Routine laboratory tests including a battery of liver function test were all within normal limits

## *Material and Methods*

Material and methods have been described in detail elsewhere [6–8] Only new data will be given herein

Factor VIII associated antigen was evaluated according to a previously reported modification of the method proposed by LAURELL [14] Anti factor VII activity was evaluated by incubating in plastic tubes at 37°C for 1 h, a mixture of patient s plasma and fresh normal plasma and a mixture of classical factor VII deficiency plasma and fresh normal plasma. Factor VII was then assayed at 60-min intervals in both mixtures The factor VII deficient plasma used in these experiments belonged to 2 patients with classical factor VII deficiency previously reported by us The factor VII level in such plasms is about 3% of normal [10] Anti factor VIII inhibitor activity was evaluated according to a modification of the method proposed by BIGGS and BIDWELL [1] A lyophilized factor VIII concentrate preparation (Kryobulin 250, Immuno Wien) was reconstituted according to the manufacturer s instructions with 30 ml of distilled water 1 ml of this preparation was then diluted 1 3 with normal saline 0 1 ml of such diluted preparation was then added to 0 9 ml of patient plasma or 'fake' plasma The fake plasma was obtained by mixing 0 2 ml of normal plasma and 0 8 ml of severe hemophilia A plasma. The factor VIII level of such mixed fake plasma was 20% of normal After a 60-min incubation period at 37°C, factor VIII was assayed in a one-stage system in both mixtures

The neutralization test was carried out according to a modification of the method proposed by LEVANON *et al* [21] The test was carried out as follows *Stage 1* anti factor VII antiserum[1] (0 05 ml) was added to 0 9 ml of the test plasma This dilution inactivated after 1 h approximately 90–95% of factor VII in normal plasma The mixture was incubated for 1 h at 37°C and then centrifuged at 5 000 rpm for 20 min. *Stage 2* 0 25 ml of the supernatant was then transferred to a second tube containing 0 25 ml of pooled normal plasma This mixture was also incubated and centrifuged in the same way as in Stage 1 *Stage 3* the supernatant was assayed for residual factor VII activity A calibration curve was constructed by diluting pooled normal plasma to have factor VII concentration over the range of 100–5%

Ristocetin platelet aggregation was carried out as previously reported [16] The final concentration of ristocetin in the cuvette was 1 5 mg/ml.

## *Results*

The coagulation study in our propositus is summarized in table I Glass clotting time was at the upper limits of normalcy Prothrombin time was slightly prolonged too and remained so even using a factor-V- and fibrinogen-

[1] The anti-factor VII antiserum used in these experiments was kindly supplied by Drs BECKER and HEINBURGER of Behringwerke Research Laboratories, Marburg (FRG)

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At the age of 6 the patient bled profusely after tonsillectomy Bleeding stopped after two local packings and a fresh blood transfusion The blood transfusion, however, had to be interrupted because of a reaction At the age of 7 the patient was involved in a car accident and sustained a fracture of the left femur and a laceration of the upper lip Bleeding from the wound was marked He was admitted to a hospital in Turin where he was sutured and a cast was applied The healing of the fracture was very slow since it required a 3 month period before the cast could be removed At the age of 8 the patient had an abdominal trauma which caused melena Bleeding subsided after a few days of bed rest At the age of 10 the patient presented excessive bleeding after a deciduous tooth extraction At that time he was suspected to have hemophilia A

At the age of 18 the patient presented severe bleeding after a molar tooth extraction He had to be admitted to a local hospital and bleeding subsided only after transfusion of fresh frozen plasma During this admission, a routine coagulation study revealed a moderate factor VII defect together with a mild prolongation of the partial thromboplastin time This latter finding remained unexplained

Easy bruising and epistaxes have been present since childhood Hematuria and hemarthrosis have never occurred At the time of study there were no bleeding manifestations

Physical examination was negative Routine laboratory tests including a battery of liver function test were all within normal limits

## *Material and Methods*

Material and methods have been described in detail elsewhere [6-8]. Only new data will be given herein

Factor VIII-associated antigen was evaluated according to a previously reported modification of the method proposed by LAURELL [14]. Anti-factor VII activity was evaluated by incubating in plastic tubes at 37°C for 1 h, a mixture of patient s plasma and fresh normal plasma and a mixture of classical factor VII deficiency plasma and fresh normal plasma. Factor VII was then assayed at 60-min intervals in both mixtures The factor VII-deficient plasma used in these experiments belonged to 2 patients with classical factor VII deficiency previously reported by us The factor VII level in such plasms is about 3% of normal [10] Anti factor VIII inhibitor activity was evaluated according to a modification of the method proposed by BIGGS and BIDWELL [1] A lyophilized factor VIII concentrate preparation (Kryobulin 250 Immuno Wien) was reconstituted according to the manufacturer s instructions with 30 ml of distilled water 1 ml of this preparation was then diluted 1 3 with normal saline, 0 1 ml of such diluted preparation was then added to 0 9 ml of patient plasma or fake plasma The fake plasma was obtained by mixing 0 2 ml of normal plasma and 0 8 ml of severe hemophilia A plasma The factor VIII level of such mixed fake plasma was 20% of normal. After a 60-min incubation period at 37°C, factor VIII was assayed in a one-stage system in both mixtures.

The neutralization test was carried out according to a modification of the method proposed by LEVANON *et al* [21] The test was carried out as follows *Stage 1* anti factor VII antiserum* (0 05 ml) was added to 0 9 ml of the test plasma. This dilution inactivated after 1 h approximately 90-95% of factor VII in normal plasma The mixture was incubated for 1 h at 37°C and then centrifuged at 5 000 rpm for 20 min *Stage 2* 0 25 ml of the supernatant was then transferred to a second tube containing 0 25 ml of pooled normal plasma This mixture was also incubated and centrifuged in the same way as in Stage 1 *Stage 3* the supernatant was assayed for residual factor VII activity A calibration curve was constructed by diluting pooled normal plasma to have factor VII concentration over the range of 100-5%

Ristocetin platelet aggregation was carried out as previously reported [16] The final concentration of ristocetin in the cuvette was 1 5 mg/ml

## *Results*

The coagulation study in our propositus is summarized in table I Glass clotting time was at the upper limits of normalcy Prothrombin time was slightly prolonged too and remained so even using a factor-V- and fibrinogen-

* The anti-factor VII antiserum used in these experiments was kindly supplied by Drs. BICKER and HEIMBURGER of Behringwerke Research Laboratories, Marburg (FRG).

*Table I* Coagulation study in the propositus

| Test | Propositus | Normal value |
|---|---|---|
| Clotting time, min | 10 | 5–10 |
| Bleeding time, min | 4 | 2–5 |
| Platelet count | 175 000 | 150 000–350,000 |
| Clot retraction | complete in 8 h | complete in less than 12 h |
| Platelet adhesiveness, % | 35 | 20–50 |
| Platelet aggregation to ristocetin (maximal amplitude of the curve), % | 65 | 70 |
| Partial thromboplastin time, sec | 52 5 | 32–42 |
| Prothrombin consumption, % | 90 | >90 |
| Thromboplastin generation test | 60 sec in 10 min | <20 sec in 6–8 min |
| Prothrombin time, sec | 17 2 | 14 |
| Stypven-cephalin clotting time, sec | 12 | 12 |
| Thrombotest sec | 53 | 43 |
| Normotest, sec | 36 | 28 |
| PP test, sec | 37 1 | 29 4 |
| Factors, II, V, IX, X, XI, XII, % | normal | 60–160 |
| Factor VII, % | 35[1] | 85–125 |
| Factor VIII, % | 18[1] | 60–160 |
| Fibrinogen, mg% | 400 | 250–500 |
| Thrombin time, sec | 19 | 18–25 |
| Euglobulin lysis time, h | 20 | 10–30 |
| Thromboelastogram r | 21 | 10–20 |
| K | 12 | 6–12 |
| ma | 52 | 50–66 |

[1] Average of several assays

containing thromboplastin Recalcification and partial thromboplastin times were moderately prolonged The partial thromboplastin time was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum or hemophilia A plasma (table II) The thromboelastogram showed a slightly prolonged 'r + K', whereas 'ma' was normal

On the contrary, Stypven-cephalin clotting time was normal Prothrombin time derivative tests (PP test, Thrombotest and Normotest) were all slightly prolonged too TG test was defective and was corrected by the substitution of patient adsorbed plasma with adsorbed normal plasma (fig 2) Prothrombin consumption was borderline (90%)

■ or ● = Factor V I and factor V I defic ency var ably symptomatic
□ or ○ Stud ed normal ⊞ or ⦶ = Not studied asymptomatic normal?
□ or ○ = Deceased asymptomatic ⌒ = Tw ns

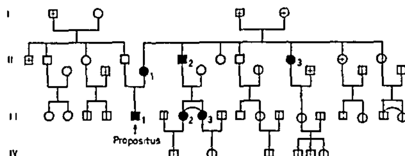



*Fig 1* Fam ly ped gree Bes des the propositus 5 other fam ly members were affected The pattern of hered tary transm ss on is autosomal dom nant All persons studied on the paternal side were normal.

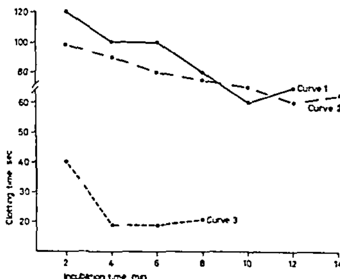



*Fig 2* Thromboplastin generation test in the propos tus Curve 1 refers to the basal curve Curve 2 was obta ned after the subst tution of pat ent s serum w th normal serum Curve 3 was obta ned after the subst tut on of pat ent s adsorbed plasma with adsorbed normal plasma

*Table II* Partial thromboplastin time (PTT) cross-correction studies in the propositus

| Mixtures (equal parts) | PTT of mixture, sec | PTT of reference plasma, sec |
|---|---|---|
| Propositus plasma | 52.5 | – |
| Propositus plasma + adsorbed normal plasma | 37 5 | – |
| Propositus plasma + normal serum | 70 1 | – |
| Propositus plasma + hemophilia A plasma | 76 | 106 |

*Table III* Factor VII and VIII inhibitors Lack of anti factor VII or anti factor VIII inhibitor in the propositus' plasma

| | Incubation time, min | | |
|---|---|---|---|
| | 0 | 60 | 120 |
| *Factor VII* | | | |
| Level of a 1 2 mixture of patient s plasma + fresh normal plasma | 80 | 86 | 84 |
| Level of a 1 2 mixture of severly factor-VII deficient plasma + fresh normal plasma | 60 | 58 | 66 |
| *Factor VIII* | | | |
| Concentrate + propositus plasma | | 70 | |
| Concentrate + fake plasma | | 78 | |

Platelet and vascular tests were all within normal limits Fibrinogen was normal and there was no hyperfibrinolysis Factor VII was moderately reduced (35% of normal) Factor VIII was moderately decreased too (18% of normal) Factors II, V, IX, X, XI and XII were all within normal limits Factor-VIII-associated antigen was at the lower limits of normalcy Factor VII protein as assayed by neutralization test was definitely higher than the factor-VII-clotting counterpart (fig 3)

No anti-factor VII or anti-factor VIII inhibitors were found in the patient's plasma (table III)

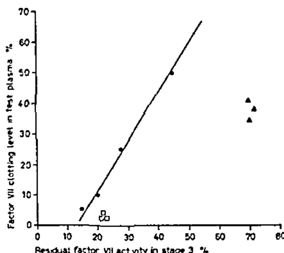



*Fig. 3* Relationship of factor VII-clotting activity and residual factor VII activity in antibody neutralization assay (stage 3) in the propositus, his mother, another affected member (▲) and in 3 patients with severe factor VII deficiency (□). The residual factor VII activity (stage 3) observed in the propositus and in the other two affected members is considerably higher than expected. The level observed is similar to that observed in normal subjects. This indicates that a normal or nearly normal factor VII antigen is present in the propositus' plasma. The dots and the best fitted line represent the calibration curve obtained by serial dilutions of pooled normal plasma.

*Table IV* Factor VII activity, factor VIII activity and factor VIII antigen in the affected family members (fig. 1) of the propositus

| Patient | Simplastin % of normal | Thromboplastin % of normal | Factor VIII activity | Factor VIII antigen |
|---|---|---|---|---|
| Propositus ($III_1$) | 40 | 31 | 18 | 60 |
| Father | 100 | 100 | 105 | 160 |
| Mother ($II_1$) | 54 | 47 | 25 | 110 |
| $II_2$ | 54 | 45 | 32 | 130 |
| $II_3$ | 65 | 64 | 26 | 100 |
| $III_2$ | 46 | 38 | 22 | 100 |
| $III_3$ | 52 | 47 | 20 | 110 |
| Normal | 85–120 | 85–120 | 60–160 | 60–160 |

Factor VII was assayed using a rabbit brain + lung thromboplastin (Simplastin) and a human brain thromboplastin (Manchester Thromboplastin). The results are the average of at least two determinations carried out on different occasions.

The daily intravenous administration of vitamin K (10 mg) for a few days failed to correct the factor VII defect

Mildly prolonged partial thromboplastin and prothrombin times due to reduced factor VII and VIII activity were found also in 5 family members including the mother Factor-VIII-associated antigen was normal in all affected patients (table IV) Factor VII antigen was assayed by means of the neutralization test in 2 patients, besides the propositus, and was normal or near normal

The patients affected turned out to be those who had presented some bleeding manifestations Only 1 patient (case $II_2$, fig 1) was affected and had so far no bleeding symptoms This patient, however, has never undergone surgery or major trauma during his life and therefore his hemostatic system has probably not been challenged so far

*Discussion*

The laboratory findings are consistent with the presence of a first- and second stage defect Specific assays demonstrated that the defect was due to the concomitant lack of factors VII and VIII The possibility our patient had von Willebrand s disease, together with a factor VII deficiency, may be safely ruled out because of the normal bleeding time, normal pletelet adhesiveness, normal factor VIII antigen and normal platelet aggregation to ristocetin The presence of normal or low-normal factor-VIII-associated antigen in the plasma of our propositus seems to suggest that the defect is of the hemophilia type This is the first demonstration of a normal factor-VIII-associated antigen in combined factor VII and factor VIII deficiency The factor VIII antigen level found in our propositus is slightly lower as compared to that found in his mother and other maternal relatives The level appears to be at the lower limits of normalcy and has remained so in repeated assays We do not know if this has any significance

It is to be remembered in this regard that a normal factor-VIII-associated antigen has been demonstrated already in all 3 patients with combined deficiency of factor V and factor VIII so far examined [*9, 17, 29*]

Our impression is that, at least as far as our family is concerned, the association between factors VII and VIII is not casual The statistical propability for such an association is indeed very low Factor VII deficiency is a rare disorder Only about 100 propable or sure cases have been described so far in the world literature and only 5 sure homozygotes have been describ-

ed in Italy together with only about 20 heterozygotes (10, 11, 22) Hemophilia A is a less rare condition and seems to be present in Italy in 1 out of 20 000 people Given a population of 55 million, approximately 3,000 hemophiliacs appear to be living at the present time in Italy It would be extremely rare for a female patient to have a factor VII defect and at the same time to be a carrier of a factor VIII defect or for a male patient to have hemophilia A and a factor VII defect Furthermore, against the causal association hypothesis remains the fact that no isolated factor VII or factor VIII deficiency was found in the family members of the maternal side of our propositus Finally, the presence of a factor VIII defect in females is not either consistent with the association of the two defects

The most likely explanation is to admit that our patients 'lack' a gene or a system of genes on an autosomal chromosome involved in the activation of both factors VII and VIII If this is so, we may suspect that they have a normal factor VII antigen too In agreement with these assumptions are the neutralization test studies The residual factor VII activity in stage 3, was defenitely higher than expected, indicating the presence of a normal or nearly normal factor VII antigen in stage-I samples In this regard, it is interesting to note that slightly higher levels of factor VII antigen as compared to factor VII activity were also present in our 3 patients with severe factor VII deficiency This is in agreement with the findings of others [3, 18, 21] Our propositus seems to have a structural abnormality of factors VII and VIII whereby a normal or near normal synthesis of the two proteins has occurred but an incomplete activation of same has ensued

The hereditary pattern in our family seems autosomal dominant No other explanation would suit the findings Therefore we would have to admit that a non von-Willebrand factor VIII defect, under certain circumstances, may be transmitted as an autosomal dominant trait

On the basis of the data presented herein it may be inferred that the family presented by CONSTANDOULAKIS [2] met fully these assumptions too In this case the mother of the propositus had a low factor VII and a prolonged glass clotting time In this pedigree 2 patients with apparent isolated mild factor VII deficiency are reported too However, factor VIII was not assayed in these latter patients and therefore a mild deficiency could have been missed On the contrary, the patient presented by GASTON *et al* [5] could represent a second type of combined factor VII and factor VIII deficiency, the casual association type In this family, in fact, the only patient affected appears to be a hemophiliac who has probably inherited one defective gene controlling factor VII from either of his parents So he appears to be a

hemophiliac and at the time a heterozygote for factor VII deficiency Unfortunately neither the father nor the mother were studied and so no data on the hereditary transmission is obtainable from this pedigree

A similar classification into two different types of defect has been recently proposed for combined factor V and factor VIII deficiency [9, 12] This latter defect, however, is transmitted as an autosomal recessive trait The data presented in this paper clearly indicate the existence of another factor VIII defect and of another factor VIII inheritance pattern

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# Book Review · Buchbesprechung · Livre nouveau

J M UNSELD **Blutersatz durch stromafreie Hämoglobinlösung** Ergebnisse tierexperimenteller Untersuchungen Springer, Berlin 1974 VII + 90 pp, 17 fig, DM 32.–/US$ 13.10

Der Autor legt die vollständige Dokumentation über seine Untersuchungen mit einer stromafreien Hämoglobinlösung am normovolämischen und hypovolämischen Zwergschwein vor Zum Vergleich diente eine Albuminlösung bzw bei den durch Blutung in den hämorrhagischen Schock versetzten Tieren die Retransfusion des Blutes Es wurde das Verhalten verschiedener Kreislaufgrössen, des $O_2$-Transportvermögens, der $O_2$ Aufnahme und der Nierenfunktion untersucht In Bestätigung früherer Befunde von RABINER erwies sich die Hämoglobinlösung nicht als nephrotoxisch Im hämorrhagischen Schock hatte sie kurzfristig eine dem Vollblut ähnliche, den Plasmaexpandern in bezug auf $O_2$ Transport überlegene Wirkung Durch die – speziesabhängige – kurze Halbwertszeit für das Verschwinden aus dem Plasma (beim Schwein ca $3^1/_2$ h) ist die Anwendbarkeit allerdings beschränkt Die Befunde werden eingehend diskutiert, wobei jedoch die neueste Literatur keine Berücksichtigung findet, da die Arbeit offenbar schon 1972 abgeschlossen worden ist Aus der Nichtberücksichtigung wichtiger älterer Arbeiten – z B derjenigen von BUNN *et al* – erklärt sich wahrscheinlich die etwas einseitige Betrachtungsweise So halten die Angaben über die Elimination der Hämoglobinlösung nicht jeder Kritik stand Ähnliches gilt für die Bemerkungen über die Sauerstoffabgabe Das kleine Buch dem eine kurze englische Zusammenfassung beigegeben ist, dürfte von jedem, der sich mit Problemen der Kreislaufregulation, des Blutersatzes und der Schockbehandlung beschäftigt, mit Interesse gelesen werden

U BUCHER, *Bern*

# Varia

Karger Medical and Scientific Publishers announce a new section on 'Inborn Errors of Metabolism in Man', to appear regularly under a special heading in the journal *Human Heredity* Articles are invited related to the metabolic aspects of the various hereditary errors of metabolism in man The editorial committee includes ANDRE DE VRIES (Petah Tikva), R W E WATTS (London), S SEGAL (Philadelphia) and ODED SPERLING (Petah Tikva) Papers should be prepared according to the rules for the preparation of manuscripts for the Karger journals, and not exceed six printed (journal print) pages Papers should be submitted to the chief editor of this new section ANDRE DE VRIES, Beilinson Medical Center, PO Box 85 *Petah Tikva* (Israel)

Acta haemat 55 193-198 (1976)

# Intravascular Fate of Granulocytes Administered by Granulocyte Transfusions[1]

G MEURET, H. J SENN, V. DE FLIEDNER and M FOPP

Department of Internal Medicine, Section of Hematology and Oncology, Kantonsspital, St Gallen

*Key Words* Autotransfusion of granulocytes Granulocyte transfusion Leukocyte kinetics

*Abstract* Granulocytes were harvested from hematologically normal individuals using continuous flow centrifugation (CFC) or filtration leukapheresis (FL) The isolated granulocytes were labeled *in vitro* by $^3$H-diisopropyl fluorophosphate ($^3$H DFP) and autotransfused Their intravascular fate was analyzed by autoradiography Immediately after autotransfusion the majority of granulocytes administered, collected in the marginal granulocyte pool Margination was particularly prominent in granulocytes isolated by FL. The distribution of transfused granulocytes between the circulating and the marginal granulocyte pool showed wide and irregular fluctuations in time Margination of transfused granulocytes was counterbalanced and its fluctuation between the two intravascular pools was stabilized by prednisone treatment The transit of transfused granulocytes from blood to tissue seemed to be governed by a random process, the half-disappearance time being either normal or prolonged Compatible granulocytes administered to hematologically normal recipients circulated for at least 20 h

Granulocyte transfusion is applied increasingly in the management of life-threatening infections during severe neutropenia [10] The cells administered with these transfusions are generally collected from normal donors using continuous flow centrifugation (CFC) or filtration leukapheresis (FL) *In vitro* studies of granulocytes harvested by these methods did not reveal any relevant morphological or functional defects [3, 7] The purpose of the present paper is to analyze their *in vivo* behavior by cell kinetic studies using *in vitro* $^3$H-DFP labeling, autotransfusion, and autoradiography

[1] Research supported by Swiss Cancer League grant No SKI FOR 034 AK 73 (1)

J M UNSELD Blutersatz durch stromafreie Hamoglobinlosung Ergebnisse tierexperimenteller Untersuchungen Springer, Berlin 1974 VII + 90 pp, 17 fig DM 32-/US$ 13 10

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# Book Review · Buchbesprechung · Livre nouveau

J. M. UNSELD: Blutersatz durch stromafreie Hämoglobinlösung. Ergebnisse tierexperimenteller Untersuchungen. Springer, Berlin 1974. VII + 90 pp., 17 fig. DM 32.–/US$ 13.10

Der Autor legt die vollständige Dokumentation über seine Untersuchungen mit einer stromafreien Hämoglobinlösung am normovolämischen und hypovolämischen Zwergschwein vor. Zum Vergleich diente eine Albuminlösung bzw. bei den durch Blutung in den hämorrhagischen Schock versetzten Tieren die Retransfusion des Blutes. Es wurde das Verhalten verschiedener Kreislaufgrössen, des $O_2$-Transportvermögens, der $O_2$-Aufnahme und der Nierenfunktion untersucht. In Bestätigung früherer Befunde von RABINER erwies sich die Hämoglobinlösung nicht als nephrotoxisch. Im hämorrhagischen Schock hatte sie kurzfristig eine dem Vollblut ähnliche, den Plasmaexpandern in bezug auf $O_2$-Transport überlegene Wirkung. Durch die – speziesabhängige – kurze Halbwertszeit für das Verschwinden aus dem Plasma (beim Schwein ca. 3½ h) ist die Anwendbarkeit allerdings beschränkt. Die Befunde werden eingehend diskutiert, wobei jedoch die neueste Literatur keine Berücksichtigung findet, da die Arbeit offenbar schon 1972 abgeschlossen worden ist. Aus der Nichtberücksichtigung wichtiger älterer Arbeiten – z. B. derjenigen von BUNN *et al.* – erklärt sich wahrscheinlich die etwas einseitige Betrachtungsweise. So halten die Angaben über die Elimination der Hämoglobinlösung nicht jeder Kritik stand. Ähnliches gilt für die Bemerkungen über die Sauerstoffabgabe. Das kleine Buch, dem eine kurze englische Zusammenfassung beigegeben ist, dürfte von jedem, der sich mit Problemen der Kreislaufregulation, des Blutersatzes und der Schockbehandlung beschäftigt, mit Interesse gelesen werden.

U. BUCHER, *Bern*

# Varia

Karger Medical and Scientific Publishers announce a new section on 'Inborn Errors of Metabolism in Man', to appear regularly under a special heading in the journal *Human Heredity*. Articles are invited related to the metabolic aspects of the various hereditary errors of metabolism in man. The editorial committee includes ANDRE DE VRIES (Petah Tikva), R. W. E. WATTS (London), S. SEGAL (Philadelphia) and ODED SPERLING (Petah Tikva). Papers should be prepared according to the rules for the preparation of manuscripts for the Karger journals, and not exceed six printed (journal print) pages. Papers should be submitted to the chief editor of this new section: ANDRE DE VRIES, Beilinson Medical Center, PO Box 85, *Petah Tikva* (Israel).

Acta haemat. 55: 193–198 (1976)

# Intravascular Fate of Granulocytes Administered by Granulocyte Transfusions[1]

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*Key Words.* Autotransfusion of granulocytes · Granulocyte transfusion · Leukocyte kinetics

*Abstract.* Granulocytes were harvested from hematologically normal individuals using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). The isolated granulocytes were labeled *in vitro* by ³H-diisopropyl fluorophosphate (³H DFP) and autotransfused. Their intravascular fate was analyzed by autoradiography. Immediately after autotransfusion the majority of granulocytes administered collected in the marginal granulocyte pool. Margination was particularly prominent in granulocytes isolated by FL. The distribution of transfused granulocytes between the circulating and the marginal granulocyte pool showed wide and irregular fluctuations in time. Margination of transfused granulocytes was counterbalanced and its fluctuation between the two intravascular pools was stabilized by prednisone treatment. The transit of transfused granulocytes from blood to tissue seemed to be governed by a random process, the half-disappearance time being either normal or prolonged. Compatible granulocytes administered to hematologically normal recipients circulated for at least 20 h.

Granulocyte transfusion is applied increasingly in the management of life threatening infections during severe neutropenia [10]. The cells administered with these transfusions are generally collected from normal donors using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). *In vitro* studies of granulocytes harvested by these methods did not reveal any relevant morphological or functional defects [3, 7]. The purpose of the present paper is to analyze their *in vivo* behavior by cell kinetic studies using *in vitro* ³H DFP labeling, autotransfusion, and autoradiography.

[1] Research supported by Swiss Cancer League, grant No. SKL FOR 034 AK 73 (1).

## Patients and Methods

The studies were carried out in 10 hematologically normal patients with malignant diseases. They all gave informed consent. Four subjects were treated with 3×10 mg prednisone per day, given orally, for the duration of the cell kinetic studies except for 1 patient, F. E. (fig. 1), who received prednisone only during the first day of the investigation. Granulocytes were collected either by the Aminco continuous flow centrifuge (American Instrument Co., Silver Spring, Md.) or by the repetitive filtration elution leukapheresis as described earlier [4]. Heparine (with FL) or heparine plus ACD (with CFC) was added to the donors' blood during cell separation while polymers such as hydroxyethylstarch or plasmagel were not applied with CFC.

The cell kinetic methods used in the present study have already been described in detail [9, 11]. In short, collected granulocytes were labeled *in vitro* by 500 μCi $^3$H diisopropyl fluorophosphate ($^3$H DFP, specific activity 5 Ci/mM) and autotransfused within 10 min. Subsequently venous blood samples were taken at suitable intervals for preparation of concentrated leukocyte smears which, after processing by autoradiography, were submitted to microscopic analysis. Granulocyte labeling indices were determined by scanning 3,000 granulocytes per sample. The circulating granulocyte pool (CGP) of the recipients was calculated from blood volume using the formula of Baker *et al.* [1] and the mean neutrophil blood count during the study. The number of transfused granulocytes in CGP after completion of autotransfusion resulted from CGP×LI/100 (where LI = 5 min posttransfusion). The number of transfused granulocytes marginating 5 min after autotransfusion was the difference between the total number of transfused labeled granulocytes and the number of circulating transfused granulocytes.

## Results

$^3$H-DFP labeling indices of transfused granulocytes ranged between 94 and 100%. The following numbers of labeled granulocytes (×10$^9$) were administered to the patients: H. H. 5.9, S. P. 6.6, F. I. 2.1, W. A. 3.2, F. E. 1.1, E. X. 2.5, G. C. 1.9, B. M. 0.9, E. A. 6.5, and L. W. 6.9. In patients not being treated by prednisone the majority of the transfused granulocytes disappeared rapidly from the circulating blood. 5 min after the autotransfusion the majority of the transfused granulocytes were located in the marginal granulocyte pool (fig. 1, 2).

With granulocytes isolated by FL, margination was more pronounced than with those isolated by CFC. In patients not being treated with prednisone, granulocyte-labeling indices scattered irregularly during the course of the investigation except in patient F. I. In this latter case, labeled granulocytes disappeared exponentially from the circulating blood with a half-

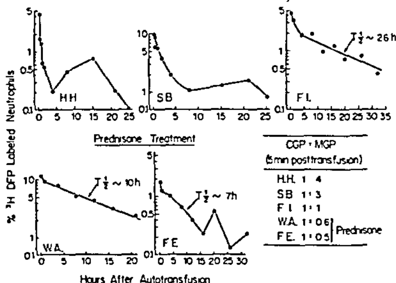



*Fig 1* Intravascular kinetics of neutrophilic granulocytes isolated by CFC, labeled *in vitro* with ³H DFP and autotransfused to hematologically normal individuals. CGP MGP indicates the distribution of the transfused granulocytes between the circulating and the marginal granulocyte pool of the recipients

disappearance time of about 26 h (fig 1) Prednisone treatment elicited a shift of transfused granulocytes to the circulating pool and caused the time course of granulocyte-labeling indices to be more regular In 3 of 4 patients, labeling indices fell in an exponential fashion, the half-times being 7, 10 and 13 h, respectively (fig 1, 2) In patient F E, scattering of granulocyte-labeling indices appeared during the second day of the study (fig 1) when prednisone treatment was discontinued In all studies labeled granulocytes administered by the granulocyte transfusion could be detected in the blood of the recipients during a period of at least 20 h

## *Discussion*

Interpretation of the results may be difficult due to possible perturbations in the steady state equilibrium of granulocyte kinetics provoked by

## *Patients and Methods*

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## *Results*

$^3$H-DFP labeling indices of transfused granulocytes ranged between 94 and 100% The following numbers of labeled granulocytes (×10$^9$) were administered to the patients H H 5 9, S P 6 6, F I 2 1, W A 3 2, F E 1 1, E X 2 5, G C 1 9, B M 0 9, E A 6 5, and L W 6 9 In patients not being treated by prednisone the majority of the transfused granulo cytes disappeared rapidly from the circulating blood 5 min after the auto-transfusion the majority of the transfused granulocytes were located in the marginal granulocyte pool (fig 1, 2)

With granulocytes isolated by FL, margination was more pronounced than with those isolated by CFC In patients not being treated with predni-sone, granulocyte-labeling indices scattered irregularly during the course of the investigation except in patient F I In this latter case, labeled gran ulocytes disappeared exponentially from the circulating blood with a half-

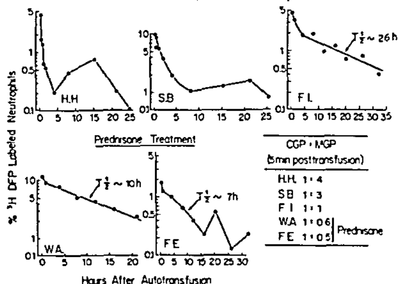



*Fig. 1.* Intravascular kinetics of neutrophilic granulocytes isolated by CFC, labeled *in vitro* with ³H DFP and autotransfused to hematologically normal individuals. CGP:MGP indicates the distribution of the transfused granulocytes between the circulating and the marginal granulocyte pool of the recipients.

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## *Discussion*

Interpretation of the results may be difficult due to possible perturbations in the steady state equilibrium of granulocyte kinetics provoked by

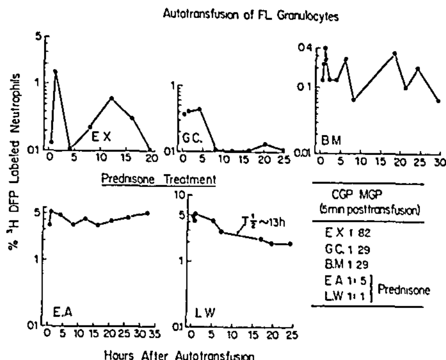



*Fig 2* Intravascular kinetics of neutrophilic granulocytes isolated by FL and autotransfused See legend of figure 1

the cell separation procedure However, to minimize this perturbation only relatively small amounts of granulocytes were harvested by CFC or FL The proportion of transfused granulocytes being detectable in circulation some minutes after completion of autotransfusion was relatively small Two arguments are in favor of the conclusion that the majority of the granulocytes resided in the marginal granulocyte pool and were not victim to phagocytosis by the reticuloendothelial system Firstly, in some of the patients the time course of granulocyte-labeling indices was irregular including high peaks This phenomenon seems to indicate a disorderly fluctuation of transfused granulocytes between CGP and MGP Secondly, prednisone treatment effected considerable enlargement of the proportion of transfused granulocytes in the CGP These considerations seem to justify the following interpretations and conclusions

(1) Granulocytes administered by granulocyte transfusions tend to accumulate in MGP Margination is more pronounced with granulocytes isolated by FL than with those isolated by CFC The difference may arise from cell interactions with the nylon fibers of the filters during cell sepa-

ration Margination of transfused granulocytes is probably the explanation for the low recovery rates reported in the literature [5, 6]

(2) The distribution equilibrium of transfused granulocytes between the two intravascular pools is unstable

(3) Margination of transfused granulocytes was effectively counterbalanced by the application of prednisone which, in addition, stabilized the distribution equilibrium of these cells between the two intravascular pools

(4) In some patients, irregular cell fluctuations between CGP and MGP did not allow for determination of the cells' disappearance curves In others, the curves followed a decaying exponential component, indicating a random transit of transfused granulocytes from the vessels into the tissue In these latter cases the half disappearance times were either in the range of normal granulocytes or were prolonged (normal range of the method 6 1–10 5 h mean 7 6 h [5]) Similar half-disappearance times were observed when allogenic neutrophils were transfused to neutropenic recipients with infections [4] The results of our cell kinetic studies in men are basically in accordance with similar observations in dogs obtained by BUCKNER *et al* [2]

(5) In hematologically normal recipients compatible granulocytes administered with granulocyte transfusions circulate for at least 20 h

In patients with agranulocytosis quantitative collections of neutrophils in the exudate of acute inflammations were carried out before and after transfusion of compatible granulocytes [4] These studies evidenced rapid accumulation and exudation of transfused granulocytes at the site of the inflammatory reaction This behavior may explain why relatively small numbers of granulocytes administered by granulocyte transfusions have proven effective in the treatment of septicemia in granulocytopenic patients [6 8]

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# Cytochemical Leukocyte Reactions in Normal Children

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*Key Words* Alkaline phosphatases Cytochemistry Esterases Leukocyte metabolism Myeloperoxydase Periodic acid Schiff reaction Sudan Black reaction

*Abstract* Five cytochemical reactions in current use (MPO PAS LAP, SB and EST) were operated on 180 children aged 1 month to 6 years, divided into three age groups (less than 12 months, 1-3 years and 3-6 years) No significant differences are apparent from one group to another, and the mean values are identical with those of adults On the other hand, certain variations are evident in comparison with the scores of newborn children important decrease in LAP, increase in SB, EST and PAS These tests should be done in investigations of inborn metabolic errors, chromosome anomalies, blood diseases, and defects in defense mechanisms.

Cytochemical reactions are becoming increasingly more important for the study of blood cells They are helpful for diagnosis of blood diseases and above all, for classifying acute leukemia For research purposes, metabolic changes which occur in various pathological states can be approached from this angle, for example, hereditary diseases These tests can be carried out with relative ease on some drops of capillary blood Such study would seem of particular interest in pediatric practice

Cytochemical leukocyte studies are numerous and deal primarily with adults, and the norm is sometimes uncertain We feel it useful to set up normal values in children and to consider the resulting variations which appear during the first years of life In a previous paper, we published the results for normal newborn children [1] The following study is concerned with children between the ages of a few weeks and 6 years

## Materials and Methods

We studied 180 children aged between a few weeks and 6 years They were all considered as normal, i e free of any clinically detectable illness They were divided into three groups

*Group 1* 40 babies were chosen at random from the Pediatric Center of the Maternity Hospital They were 18 boys and 22 girls, all over 1 month and less than 12 months old

*Group 2* A blood sample was taken from 57 children (34 boys and 23 girls aged 1–3 years, at a nursery in town

*Group 3* 83 samples were obtained from children aged 3–6 years who came from a kindergarden They were 43 boys and 40 girls

This division into three age groups did not depend on known or suspected physiological variations, it was simply a result of the method of recruitment

Five cytochemical tests were used They were carried out on blood smears obtained from finger prick samples At the same time, a hemogram was performed For each cytochemical reaction a score based on 100 cells was established and grades assigned according to the intensity of the reaction

*Myeloperoxydase (MPO)* GRAHAM KNOLL technique [2] modified by shortening the reaction time (75 sec instead of 5 min) The score ranged from 0 to 300

*Leukocyte alkaline phosphatase (LAP)* α Naphtyl phosphate technique followed by diazoreaction according to a modification in the Gomori method [3] The Kaplow score had a theoretical variation from 0 to 400

*Non specific esterase (EST)* WACHSTEIN and WOLF technique [4] modified by GROZDEA *et al* [5] We chose a score varying between 0 and 300

*Polysaccharides by periodic acid Schiff reaction (PAS)* McManus technique modified by lengthening the reaction time and omitting rinsing in sulfurous medium Two scores were used 0–300 for polymorphonuclear and 0–300 for lymphocytes

*Lipids by Sudan Black (SB)* Technique of HAYOHE *et al* [6] The score varied from 0 to 400

## Results

The results of the 5 reactions in each age group are shown in table I The number of subjects studied in each group varies from reaction to reaction, this is mainly due to technical problems (reaction failure, illegibility due to the poor quality of the smear or to morphological leukocyte changes)

## Comments

Blood leukocytes play a vital role in specific or nonspecific defense of the body against pathogenic agents These cells use general cellular meta-

*Table I* Results of cytochemical leukocyte reactions in children of three age groups

| Reactions[1] | Number of subjects | Grading | | | | | Scores | Standard error |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | | |
| *Children less than 12 months old* | | | | | | | | |
| SB | 41 | 0.00 | 0.02 | 4.61 | 68.88 | 26.49 | 321.83 | 19.16 |
| MPO | 33 | 0.45 | 5.88 | 85.24 | 6.91 | | 197.09 | 19.95 |
| PMN PAS | 44 | 2.43 | 20.91 | 71.50 | 4.77 | | 178.23 | 32.78 |
| LAP | 44 | 29.14 | 25.18 | 24.34 | 20.34 | 1.00 | 138.89 | 46.49 |
| EST | 20 | 0.31 | 29.72 | 55.72 | 14.33 | | 180.00 | 29.51 |
| L-PAS | 44 | 86.80 | 8.73 | 3.84 | 0.61 | | 18.25 | 10.39 |
| *Children aged 1–3 years* | | | | | | | | |
| SB | 53 | 0.00 | 0.00 | 1.71 | 69.92 | 28.28 | 326.49 | 13.95 |
| MPO | 41 | 0.05 | 2.12 | 80.22 | 17.61 | | 215.39 | 14.38 |
| PMN PAS | 53 | 0.00 | 17.53 | 77.87 | 4.58 | | 187.02 | 15.39 |
| LAP | 52 | 15.15 | 21.17 | 29.54 | 32.27 | 1.87 | 184.52 | 50.97 |
| EST | 20 | 0.00 | 24.25 | 29.50 | 16.25 | | 192.00 | 27.59 |
| L PAS | 53 | 80.40 | 15.58 | 3.89 | 0.13 | | 23.75 | 14.95 |
| *Children aged 3–6 years* | | | | | | | | |
| SB | 82 | 0.00 | 0.02 | 0.62 | 74.02 | 25.45 | 325.15 | 12.72 |
| MPO | 80 | 0.22 | 6.04 | 86.55 | 7.19 | | 200.71 | 15.67 |
| PMN PAS | 81 | 0.01 | 14.54 | 81.28 | 3.67 | | 188.11 | 17.23 |
| LAP | 75 | 22.09 | 21.83 | 25.31 | 29.20 | 1.49 | 166.24 | 54.98 |
| EST | 20 | 0.35 | 29.35 | 57.10 | 13.20 | | 183.15 | 33.14 |
| L PAS | 82 | 80.93 | 16.73 | 2.26 | 0.09 | | 21.50 | 9.87 |

[1] SB = Sudan Black, MPO = myeloperoxidase, PMN PAS = polymorphonuclear periodic acid Schiff reaction, LAP = leukocyte alkaline phosphatase, EST = non specific esterase, L PAS = lymphocyte periodic acid Schiff reaction

bolic ways and it may be interesting to study them for research purposes in genetic metabolic diseases [7].

The five tests used in this work were chosen for their practical interest, and particularly for cytological diagnosis of malignant blood diseases. They are quite easily carried out and enable one to approach metabolic cell activity. Thus MPO plays a fundamental role in bactericidal activity of phagocytes [8]. Lipids, some of which are shown by Sudan Black,

*Table II* Comparison of mean scores for the five cytochemical reactions in full born babies and adults

| Reactions[1] | Age group | | | | adults | |
|---|---|---|---|---|---|---|
| | newborns | < 12 months | 1–3 years | 3–6 years | males | females |
| SB | 299 | 322 | 326 | 325 | – | – |
| MPO | 195 | 197 | 215 | 201 | 215 | 218 5 |
| PMN PAS | 177 | 178 | 187 | 188 | 168 50 | |
| LAP | 220 | 139 | 184 | 166 | 105 5 | 155 |
| EST | 141 | 180 | 192 | 183 | – | – |
| L-PAS | 9 | 18 | 24 | 22 | 22 5 | |

[1] For abbreviations, see table I

make up part of the cellular membrane and constitute an important reserve of energy, together with MPO, they may play a part in defense against bacterial infection The PAS positive component of granulocytes is essentially glycogen which, in its intracytoplasmic accumulation, constitutes an important reserve of energy for phagocytosis On the other hand, the quantities contained in lymphocytes are too small to be shown by PAS reaction, this explains their very low scores The precise role of esterases remains unclear, they may take part in metabolic events occurring during phagocytosis [9] LAP, of which several isoenzymes are known, participates in glucose and lipid metabolism and in cell division Their production is controlled by sex hormones [10] and their level increases during infection This would seem to show the interest of performing these three cytochemical reactions (MPO, PAS, LAP) as part of an investigation of nonspecific cellular defense mechanisms As these elements are under genetic control, the study of cytochemical reactions would seem justified for hereditary chromosomal anomalies

No significant variation can be seen between age groups in these five tests, however, the highest mean values are always found in the age group 1–3 years The different score levels are similar to these of adults for PAS, LAP and MPO On the other hand, the results differ from those of full-born babies (table II) Three types of evolution can be noted during the first month (1) an important decline in LAP, (2) no PMN-PAS or MPO variation, (3) an increase in SB, EST and L-PAS

The following remarks may be made about these results The LAP variations may depend on neonatal hormonal changes The absence of modifications in granulocyte PAS and MPO would seem to suggest that leukocyte (functional) activity is identical whatever the age As for the increase of PAS in lymphocytes between the time of birth and 3 years, the only possible parallel is with the acquisition of immune information which is highest at this age

The difficulty arises for the comparison of our results with others as very few publications cover these problems To our knowledge, the only precise work is that of KATATA [11] on LAP In any case, this author has not used the same scoring as we did, alone the general aspect of our results may be compared he found the same increase in LAP at birth and lower scores for infants of 9–10 months after which the scores rose again up to a slightly higher level than those of adults

The use of the cytochemical test on leukocytes offers two points of interest For basic blood research, they give information about certain aspects of cell metabolism and their study in chromosome anomalies can clarify ideas about certain substrata and enzyme regulation In practice, they can be of particular help for the diagnosis of some inborn metabolic errors, of blood diseases such as acute leukemia and susceptibility to infection This work enables normal scores to be set up for the five reactions used in children during the first 6 years, which allows a better interpretation of the results in children

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# Elektronenmikroskopische Lokalisation von Thiaminpyrophosphatase und Nucleosiddiphosphatase in Lymphozyten

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*Key Words* Lymphocyte enzymes · Phosphatases · Ultrahistochemistry

*Abstract* In ductus thoracicus lymphocytes of the rat the localization of specific phosphatases was studied by means of ultrahistochemistry. Thiaminepyrophosphatase was found in the outer lamellae of the Golgi field as well as in lysosomes whereas nucleosiddiphosphatases could be localized on the plasma membrane at low pH. These findings are correlated with the phaenotype of B- and T-lymphocytes. Their functional significance is discussed.

Lichtmikroskopische enzymhistochemische Untersuchungen an Zellen der lymphatischen Organe von Mensch und zahlreichen Saugern [17, 18, 35] lassen erkennen, dass der Gehalt an phosphatspaltenden Enzymen sowohl spezies- als auch organabhängig ist. In der menschlichen Milz zeichnen sich T-zellabhängige und B-zellabhängige Regionen durch den Besitz charakteristischer Enzyme aus [24]. Periphere Lymphozyten scheinen dagegen nur sehr spärlich mit nachweisbaren Enzymaktivitäten ausgestattet zu sein [1, 5, 7, 17]. Während beim Meerschweinchen Thymozyten in der Thymusrinde eine hohe Aktivität der alkalischen Phosphatasen besitzen, konnte dieses Enzym in peripheren T-Lymphozyten nicht mehr nachgewiesen werden [17, 32].

Eine deutliche Abhängigkeit vom Differenzierungsstadium der Lymphozyten weist auch die ATPase auf. Plasmoblasten sind durch eine höhere Enzymaktivität gekennzeichnet als Plasmazellen und nicht aktivierte B-Lymphozyten [12, 28, 37]. Spezifische Phosphatasen, wie die Thiaminpyrophosphatase (TTPase) oder Nucleosiddiphosphatasen (Inosiddiphosphatase = IDPase, Uridindiphosphatase = UDPase, Guanosidinphosphatase = GDPase) sind eng mit dem Golgi-Feld bzw. mit dem Plas-

malemm assoziiert An stimulierten Lymphozyten aus dem Lymphknoten des Meerschweinchens konnte eine TPPase-Aktivitat in den inneren Lamellen des Golgi-Feldes nachgewiesen werden [4] Fruhere Untersuchungen an Ductus-thoracicus(DT)-Lymphozyten des Hundes haben eine Enzymaktivitat vornehmlich an der konvexen Seite der Dictiosomen ergeben [11] Da das Golgi-Feld eine wichtige Rolle bei der Glykoproteinsynthese spielt und Membranen des Golgi-Feldes teils als Komponenten in die Zellmembran ausgeschleust werden, kommt dem Golgi-Apparat kleiner Lymphozyten mit Hinblick auf ihre Oberflachenspezialisierung eine wesentliche Bedeutung zu

## *Material und Methode*

Zur Untersuchung gelangten Lymphozyten aus dem DT der Ratte Die DT Drainage wurde nach BOLLMANN *et al* [6] durchgeführt Die Lymphozyten wurden in 1 5% Glutaraldehyd in 0 1 M Cacodylatpuffer (380 mosm) 20 min bei 4 °C fixiert, anschliessend zentrifugiert und das Pellet zweimal in Puffer gewaschen Der Nachweis der TPPase bzw NDPase erfolgte im Medium nach NOVIKOFF und GOLDFISCHER [26] fur 2 h bei 37 °C Medium TPP 2 mM bzw UDP, $MnCl$ 5 mM, Tris maleatpuffer 0 2 M pH 5 0–7,5, Pb $(NO_3)_2$ 3,6 mM Das Medium wurde vor Gebrauch auf 37 °C erwarmt und filtriert Uranylcitrat in 0 01 M Konzentration wirkte als Inhibitor Nach der Inkubation wurde das Pellet in 0,1 M Cacodylatpuffer gewaschen mit 1% gepufferter $OsO_4$ nachfixiert, über Aceton entwassert und in Epon/Araldit Gemisch eingebettet Die Ultradunnschnitte wurden teilweise mit Uranyl acetat nachkontrastiert

## *Befunde*

In kleinen Lymphozyten aus dem DT beschrankt sich der Nachweis eines TPP spaltenden Enzyms im wesentlichen auf das Golgi-Feld Zusatzlich weisen Lysosomen Reaktionsprodukte auf (Abb 1) In der Regel sind die ausseren konvexen 1–2 Golgi-Zisternen markiert (Abb 2a), wahrend die Innenzisternen des ohnehin schmalen Golgi-Feldes frei bleiben Der Niederschlag ist aber auch innerhalb der markierten Membranstapel nicht gleichmassig verteilt Haufig wird sehr dickes Prazipitat in der aussersten Zisterne gefunden oder in den kolbenformig erweiterten Endabschnitten der einzelnen Membranabschnitte (Abb 2b) Andere Teile des Golgi-Feldes weisen sehr schwaches und locker verteiltes Reaktionsprodukt auf

Selten findet man Golgi-Felder, die eine genau umgekehrte Markierung der einzelnen Zisternen zeigen Hier liegt ein starker Niederschlag

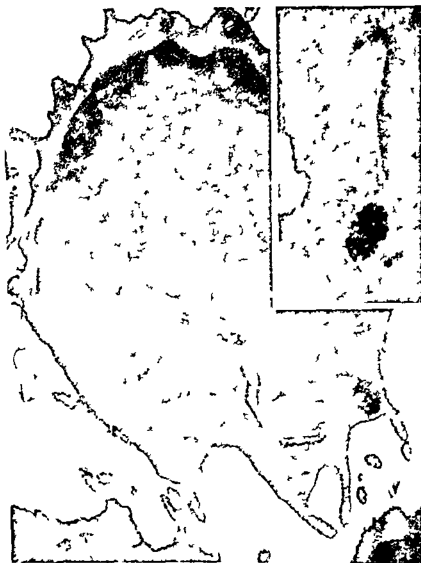


*Abb 1* Kle ner Lymph zyt m t TPPase Reakt on in der au seren Lamelle des C lg Feldes. Das Pla malemm t t negat v Z rka 30 000 Ausschn tt Lysosom m t pos t er Reakt on auf Tl Pase Z ka 40 000

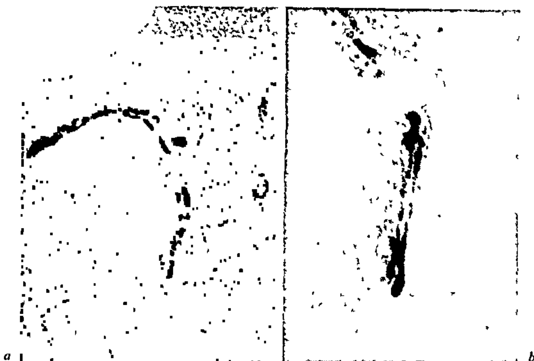


*Abb* 2. *a* TPPase Aktivität in den konvexen Lamellen des Golgi-Feldes Zirka × 50 000 *b* Kräftige TPPase-Reaktion in kolbenförmigen Endauftreibungen der Golgi-Lamellen Zirka × 57 000

in den inneren konkaven Elementen (Abb. 3a, b). Verändert man den pH-Wert der Inkubationslösung, so ergibt sich ein deutlicher Abfall des Reaktionsproduktes im sauren Bereich; meistens ist der Nachweis negativ. Eine Inkubation ohne Substrat lässt ebenfalls keinen spezifischen Niederschlag entstehen. Schwache Präzipitatbildungen im Zytoplasma, *ohne dass eine Zuordnung zu bestimmten Zellstrukturen möglich ist, sind* als unspezifische Hydrolyseprodukte anzusehen Sie treten in gleicher Verteilung auch nach Inkubation ohne Substrat auf. Tauscht man im Medium das Substrat TPP gegen UDP aus, so tritt bei pH 7,2 im Golgi-Feld ein Niederschlag auf, der aber sehr locker in den einzelnen Zisternen verteilt ist. Zusätzlich ist an der Zellmembran schwaches Reaktionsprodukt zu finden (Abb. 4) Die Reaktionsstärke an der Zellmembran nimmt mit fallendem pH zu und ist bei pH 5,5 am kräftigsten In gleichem Masse wie die Aktivität an der Plasmamembran zunimmt, kommt es im Golgi-Feld zu einem negativen Ausfall der Reaktion, so dass bei pH 5,5 eine UDPase-Aktivität nur noch an der Zellmembran lokalisierbar ist (Abb 5).




*Abb 3* TPIase Akt tat n den konkaven Lamellen des Golg Fel a Z rka 35 000 *b* Zirka 9( 00




*Abb. 2. a* TPPase-Aktivität in den konvexen Lamellen des Golgi-Feldes. Zirka × 50 000. *b* Kräftige TPPase-Reaktion in kolbenförmigen Endauftreibungen der Golgi-Lamellen. Zirka × 57 000

in den inneren konkaven Elementen (Abb. 3a, b). Verändert man den pH-Wert der Inkubationslösung, so ergibt sich ein deutlicher Abfall des Reaktionsproduktes im sauren Bereich, meistens ist der Nachweis negativ. *Eine Inkubation ohne Substrat lässt ebenfalls keinen spezifischen* Niederschlag entstehen. Schwache Präzipitatbildungen im Zytoplasma, ohne dass eine Zuordnung zu bestimmten Zellstrukturen möglich ist, sind als unspezifische Hydrolyseprodukte anzusehen. Sie treten in gleicher Verteilung auch nach Inkubation ohne Substrat auf. Tauscht man im Medium das Substrat TPP gegen UDP aus, so tritt bei pH 7,2 im Golgi-Feld ein Niederschlag auf, der aber sehr locker in den einzelnen Zisternen verteilt ist. Zusätzlich ist an der Zellmembran schwaches Reaktionsprodukt zu finden (Abb. 4). Die Reaktionsstärke an der Zellmembran nimmt mit fallendem pH zu und ist bei pH 5,5 am kräftigsten. In gleichem Masse wie die Aktivität an der Plasmamembran zunimmt, kommt es im Golgi-Feld zu einem negativen Ausfall der Reaktion, so dass bei pH 5,5 eine UDPase-Aktivität nur noch an der Zellmembran lokalisierbar ist (Abb. 5).



*Abb. 3.* TPPase-Aktivität in den inneren konkaven Lamellen des Golgi-Feldes. *a* Zirka 35 000, *b* Zirka 90 000

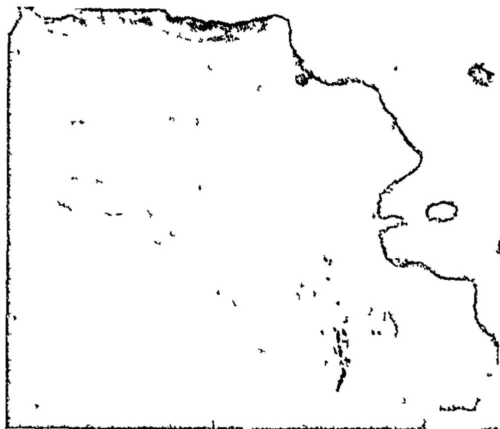


*Abb 4* UDPase Aktivität im Golgi Feld und an der Plasmamembran bei pH 7 2 Zirka × 36 000

## *Diskussion*

Die DT Lymphozyten der Ratte setzen sich aus zirka 85% T Lymphozyten und 12–15% B Lymphozyten zusammen [29] Zieht man den Phänotyp der peripheren B Zelle und T-Zelle, wie sie im Scanningmikroskop von POLLIACK *et al* [27] beschrieben worden sind, zur Identifizierung der TPPase und NDPase positiven Lymphozyten heran so fällt auf, dass TPPase positive Lymphozyten im Schnittbild immer eine mit sehr vielen fingerformigen Fortsätzen versehene Zellmembran besitzen (Abb 1) Dies ist ein Merkmal fur B Lymphozyten [27] Demgegenuber sind NDPase positive Zellen uberwiegend kugelige Lymphozyten ohne starke Gliederung der Zellmembran (Abb 5) entsprechend den T-Zell-Merkmalen [20 21 27] Ob diese Befunde an definierten B bzw




*Abb. 5.* UDPase-Aktivität bei pH 5,5 ist nur an der Plasmamembran zu finden, während das Golgi-Feld (GO) frei bleibt. Zirka × 30 000

T-Lymphozyten erhärtet werden können, wird zurzeit noch untersucht. Ihre Interpretation gestaltet sich aufgrund der Problematik, die ein histochemischer Nachweis von TPPase und NDPase mit sich bringt [2, 3, 13, 15, 23] sicher nicht zufriedenstellend, jedoch mögen einige spekulative Deutungen erlaubt sein.

Die histochemische Lokalisation der TPPase im Golgi-Feld vieler Sauger- und Pflanzenzellen macht dieses Enzym zu einem zytochemischen Marker des Golgi-Feldes [9] Die hohe Aktivitat der TPPase in stark sekretorisch tatigen Zellen konnte darauf hinweisen, dass Diphosphatasen allgemein entweder an der Ausschleusung oder an einigen Schritten der Synthese von Sekreten beteiligt sind Bei der Bildung der Glykolipide und der Glykoproteine, die als Membranbausteine oder in der Glykokalyx Verwendung finden oder aber als Antikorper ausgeschleust werden, wird eine Beteiligung der Diphosphatasen diskutiert [14, 34] Die Lymphozytenoberflache ist eine sehr dynamische Membran, an der die Umsatzraten der einzelnen Bausteine im Durchschnitt wenige Stunden betragen [19] Bei Milz-B-Lymphozyten waren innerhalb von 6 h mehr als 40% der Oberflachenimmunglobuline ausgetauscht [36] MELCHERS und ANDERSSON [22] haben nachweisen konnen, dass sich in B-Lymphozyten zirka 90% des neugebildeten IgM nach 4 h noch in der Zelle befanden Nach einer Verzogerung von 1 h begann die Ausschleusung, und nach weiteren 4 h war ebenfalls mehr als die Halfte des IgM von der Lymphozytenmembran abgegeben

Neben der Bereitstellung von Immunglobulinen als Oberflachenrezeptoren von B-Zellen steht die Produktion anderer Glykoproteine und Glykolipide fur die Oberflachenschicht sicher an weiterer dominierender Stelle, zumal dieser Teil dem Lymphozyten seine wichtige biologische und immunkompetente Stellung verleiht [38] Diese Produkte werden uber Golgi-Vesikel an die Plasmamembran herangefuhrt und dann entweder in das umgebende Milieu entlassen oder in die Plasmamembran mit eingebaut [36] Dieser Vorgang erfordert eine standige Membranneubildung, und deswegen wird das Golgi-Feld von einer Reihe von Autoren auch als Ort des Phospholipidmetabolismus angesehen [8, 33] TPP-hydrolysierende Enzyme sollen dabei die Menge des Acetyl-CoA beeinflussen, das in Membranphospholipide eingeschleust wird Auch hierfur konnte die TPPase-Aktivitat in B-Lymphozyten Ausdruck sein

Die vorliegenden Befunde uber Lokalisation von TPPase in Golgi-Feld und Lysosomen und von NDPase an der Zellmembran sprechen dafur, dass es sich hier um zwei verschiedene Enzyme handelt Hierbei ist die pH-Abhangigkeit der NDPase von besonderem Interesse Wahrend bei neutralem pH im Golgi-Feld eine leichte NDPase-Aktivitat auftritt, verschiebt sich der Reaktionsort im sauren Bereich zugunsten der Aktivitat an der Plasmamembran Nimmt man an, dass ein Enzym nur ein pH-Optimum besitzt, so konnte es sich bei der NDPase des Golgi-

Feldes und der des Plasmalemms um zwei verschiedene Enzyme handeln die beide NDPase spalten konnen Diese Vorstellung wird auch dadurch bekraftigt, dass unterschiedliche Funktionen etwa als Synthetasen und Transferasen angenommen werden [10] Eine weitere Erklarung konnte darin zu sehen sein, dass die Aktivitat im Golgi Feld, als dem Produktionsort vieler Enzyme, eine Reaktion *in statu nascendi* ist die Hauptaktivitat aber erst an der Zellmembran entfaltet wird

Die Bedeutung von Glykosyltransferasen fur die Zelladhasion und fur das gegenseitige Erkennen von Zellen darf als gesichert gelten Hierbei spielen Nucleosidasen, vor allem UDPase, eine wichtige Rolle [16, 30, 31] Zwar stehen fur den Nachweis der Glykosyltransferasen keine histochemischen Methoden zur Verfügung doch kann die Lokalisation von Nucleosidasen unter Umstanden als indirekter Nachweis fur Glykosyltransferasen aufgefasst werden Dass T-Lymphozyten eine deutliche NDPase-Aktivitat an der Zellmembran aufweisen, erscheint sinnvoll, da gerade die T-Lymphozyten permanent mit der Diskriminierung von eigenen und nicht eigenen zellularen Antigenen befasst sind

Ahnlich wie die funktionelle Bedeutung der unspezifischen Esterase von T-Lymphozyten noch unklar ist [25] mussen fur die NDPase weitere funktionelle Moglichkeiten untersucht werden z.B ihre Rolle bei der endozytotischen Aufnahme von Antigen oder bei dem zytotoxischen Effekt von aktivierten T-Zellen auf ihre Target Zellen

## *Zusammenfassung*

An DT Lymphozyten wird die elektronenmikroskopische Lokalisation von TPPase und UDPase untersucht Hierbei zeigt sich dass die TPPase im Golgi Feld und in Lysosomen lokalisiert werden kann wahrend UDPase abhangig vom pH Wert im Golgi Feld und an der Plasmamembran gefunden werden kann Die funktionelle Bedeutung der nachgewiesenen Enzyme bei B und T Zellen wird diskutiert

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# Impairment of Platelet Adhesiveness and Platelet Factor 3 Activity in Cyanotic Congenital Heart Disease

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*Key Words.* Congenital heart disease · Haemostasis · Platelet adhesiveness · Platelet factor 3 · Platelet function · Polycythemia

*Abstract.* In 33 children with cyanotic congenital heart disease the platelet function has been studied. The most significant changes were reduced platelet adhesiveness to glass and impaired availability of platelet factor 3 in nearly 50% of the patients. Although clot retraction was poor in 84% of them, thrombocytopenia and prolonged bleeding time were not significant features.

Bleeding diathesis in cyanotic congenital heart disease (CCHD), leading to severe and even fatal postoperative haemorrhage has been recognised for some time. A wide variety of haemostatic abnormalities have been reported to be present preoperatively. Thrombocytopenia [12, 20, 24], defective clot retraction [1, 7, 16], prolonged prothrombin time [15, 16, 18, 24], prolonged partial thromboplastin generation time [1, 5, 17], hypofibrinogenaemia and accelerated fibrinolysis [12, 20] have been noted. Recently, defective aggregation of platelets to ADP or collagen has been recognised [11, 13, 22]. To our knowledge, in none of the available studies impairment of platelet adhesion or platelet factor 3 activity has been found. The purpose of this communication is to describe these platelet abnormalities in CCHD. Coagulation defects were excluded by appropriate laboratory tests.



*Fig 1* Apparatus for measuring platelet adhesiveness to glass

## *Materials and Methods*

33 patients of both sexes were evaluated Their ages varied from 1 to 12 years The diagnoses included Fallot s tetralogy, total anomalous pulmonary venous drainage, tricuspid atresia and transposition of great vessels None of the patients displayed evidence of abnormal bleeding at the time of study They had not received aspirin or any other drug known to alter platelet function for at least 10 days prior to study None of the patients had evidence of renal or liver disease

For tests of coagulation and platelet function venous blood was collected in siliconised syringes and 9 vol added to 1 vol of 3 2% trisodium citrate The amount of citrate was adjusted proportionally to the volume of packed red cells (VPRC) in polycythaemic subjects with VPRC above 45%, using the formula

x = 100 – VPRC (patient) 55

where x = volume of citrate in ml to which whole blood is added to a final volume of 10 ml Each patient was studied with an age matched control

The methodologic difficulties involved in separating platelet rich plasma (PRP) to study platelet function in thrombocytopenic children with polycythaemia are formidable Insufficient quantities of PRP obtained resulted in an unequal number of tests in some instances

Haemoglobin was determined as cyanmethaemoglobin using Drabkin s solution VPRC was determined by Wintrobe s method Routine platelet counts were performed with ammonium oxalate as the diluent according to HARDISTY and INGRAM [9] Bleeding time was measured by Ivy s method [4] and clot retraction according to BIGGS and MACFARLANE [2]

Platelet adhesiveness to glass was tested *in vitro* by a modified Salzman s technique The apparatus was assembled at the National Physical Laboratory Delhi (fig 1) Blood from a clean venipuncture was allowed to flow through a glass column 9 cm long containing 2.5 g of glass beads of 0 5 mm diameter each at a constant pressure of 100 mm Hg for a period of 20 sec in each instance Platelet aggregation to ADP was measured according to HARDISTY and INGRAM [9] and to collagen by the method of ZUCKER and BORRELLI [26] Platelet factor 3 (Pf 3) availability was determined by the method based on kaolin clotting time of dilutions of PRP as described by HARDISTY and HUTTON [10]

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*Table I* Frequency of platelet defects in cyanotic congenital heart disease

| Test | Number of patients investigated | Patients showing the defect | |
|---|---|---|---|
| | | number | % |
| *Platelet count* | *30* | *5* | *16 6* |
| Bleeding time | 28 | 3 | 10 7 |
| Clot retraction | 32 | 27 | 84 3 |
| Platelet adhesiveness to glass | 28 | 13 | 46 4 |
| Platelet aggregation to | | | |
| ADP | 27 | 0 | 0 |
| Collagen | 11 | 5 | 46 0 |
| Pf 3 activity | 24 | 12 | 50 0 |

*Table II* Severity of platelet defects in cyanotic congenital heart disease (mean±SD)

| Test | Patients | Controls | p | t |
|---|---|---|---|---|
| Platelet count $\times 10^3/\mu l$ | 232 0±86 0 | 368 9±84 7 | | |
| Bleeding time min | 3 7±2.0 | 2 3±1 3 | | |
| Clot retraction % | 25 7±12 8 | 44 7±5 8 | <0 0005 | 2 64 |
| Platelet adhesiveness % | 30 0±15 9 | 39 0±4 8 | <0 01 | 2 64 |
| Platelet aggregation with | | | | |
| ADP sec | 10 2±5 0 | 8 8±4 6 | >0 05 | 0 828 |
| Collagen % | 35 0±19 0 | 39 0±8 5 | >0 05 | 0 64 |
| Pf 3 availability, % | 4 7 | >25 | | |

## *Results*

In the 33 patients studied, haemoglobin below 9 g% was seen only in 9, in the remaining it ranged between 13 and 22 g% 17 children showed VPRC of 50% and above The incidence of abnormal haemostasis was 100% In 25 of the 33 children, two or more haemostatic abnormalities were detected Platelet dysfunction was isolated in 11 patients while in 18 it coexisted with coagulation defects The abnormal haemostatic profile was characterised by impairment of clot retraction, reduced platelet adhesion to glass and poor availability of Pf-3 (table I) As many as 27 of the 33 patients investigated showed poor syneresis of the clot Amongst these

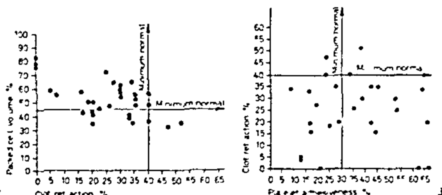


*Fig 2* Relation of packed cell volume to clot retraction
*Fig 3* Relationship between clot retraction and platelet adhesiveness.

27, 4 were thrombocytopenic and 14 had VPRC above 50%. Nearly half of the patients showed impaired platelet adhesiveness and aggregation to collagen Almost an equal number exhibited poor availability of Pf-3 In none was the platelet aggregation to ADP defective Thrombocytopenia or prolonged bleeding time were not significant features, being present only in 3 and 5, respectively, of the 28 and 30 patients investigated

The severity of the platelet defects in cyanotic children is summarised in table II Analysed statistically, the significant abnormalities comprised impaired clot retraction, reduced platelet adhesion to glass, and poor availability of Pf-3 Platelet count, bleeding time and aggregation of platelets to ADP were not different from those of controls Likewise, an apparent decrease in platelet aggregation to collagen was statistically not significant

Since anoxic polycythaemia of varying degree is a common denominator to all patients with cyanotic variety of congenital heart disease, the haemostatic defects were correlated with VPRC Patients who suffered from multiple haemostatic defects had significantly higher median VPRC than those with a single defect ($p<0.05$, $t = 1.84$) While none amongst the latter had VPRC greater than 60%, it was the case at least in 7 children who had several haemostatic defects

In general the higher the VPRC, the poorer was the clot retraction A correlative study between the two (fig 2) revealed that of the 27 children

who showed impaired retraction of the clot, 19 had VPRC more than 45% Of the latter, in every instance the clot retraction was poor

No relationship, however, existed between platelet adhesion to glass and the VPRC In 19 patients with VPRC above 45%, adhesiveness of platelets to glass was impaired in 10 and normal in 9 In fact, in 10 of the 13 children it was decreased in spite of the raised VPRC On the other hand, at least in some of the patients, platelet adhesiveness seemed to influence the clot retraction Thus, in 13 children the platelet adhesion was subnormal, and in all except two the clot retraction was also poor (fig 3) However, an equal number of patients did not show any impairment in platelet adhesiveness although their clot retraction was decreased

## *Discussion*

Our results demonstrate a high incidence of haemostatic defects in children suffering from CCHD The study further brings out the role of platelet dysfunction in postoperative bleeding so frequently observed in this disorder A defect of platelet function was demonstrable in nearly 60% of the cases In one third, it constituted the sole abnormality Impaired adhesion of platelets to glass and decreased availability of Pf-3 were characteristic changes We are not aware of any study describing these changes in CCHD The only platelet dysfunction recognised so far is defective aggregation to ADP or collagen

HELLEM *et al* [14] observed a direct correlation between the platelet adhesiveness and the VPRC We failed to find such a correlation On the contrary, in half of the patients showing high VPRC, the platelet adherence was reduced The reason for this is not clear A concomitant decrease in availability of Pf-3 in these children suggests that there may be a platelet membrane defect, inhibiting platelet reaction to greater concentrations of ADP released from a larger red cell mass

A number of physiological and biochemical changes in aging platelets have been reported in the past Old platelets as compared to young were less adhesive to glass [23, 25] and to collagen [15] Two independent studies indicate that old platelets were less effective in clot retraction [6, 21] Recently, JOHNSON *et al* [17] found aged platelets to be associated with long bleeding times, decreased platelet adhesiveness *in vivo* and *in vitro* as well as poor availability of Pf-3 Thus, it would be not unreasonable to assume that a premature aging of platelets brought about by a per-

sistent anoxic state in CCHD patients may be responsible for the abnormalities of platelet function found in these children

A normal aggregation of platelets to ADP was not accompanied by normal release of Pf-3, as could have been expected This divergence can best be explained by the fact that in the Pf-3 test used, kaolin contact and not aggregation was the agent triggering release of platelet contents However, there is evidence to suggest that total phospholipid content of aging platelets may indeed be less [8, 19]

Poor clot retraction chracterised 84% of the cases It is well known that changes in the platelets or increase in the red cell mass significantly impair the ability of the clot to retract [3] Considering that these children showed negligible thrombocytopenia, the defective clot retraction would appear to be due to an increase in the red cell mass Raised VPRC in 70% of the children showing impaired clot retraction would distinctly seem to favour this possibility

Eleven of the 13 children showing poor clot retraction also exhibited abnormal platelet adhesiveness This suggests that poor platelet adhesion may be an additional factor causing impairment of clot retraction If so, its mechanism may be related to defective release of thrombosthenin from a decrease in platelet adhesion

Since none of the children showed bleeding preoperatively, it would appear that an altered platelet function together with deficient coagulation factor(s) may result in a basically incompetent haemostatic status which fails to maintain an equilibrium postoperatively Appropriate screening tests performed preoperatively can detect the bleeding diathesis and thereby help in its management

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# Growth of Haematopoietic Cells of Mouse Fetal Liver in Diffusion Chambers

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*Key Words* Culture of haematopoietic cells Diffusion chamber Erythroblasts Fetal haematopoiesis Granulocytes Macrophages

*Abstract* Growth of haematopoietic cells from fetal mouse liver of 14 to 16 day gestation was studied with the diffusion chamber technique The culture period varied from 1 to 8 days Cell proliferation and haemoglobin synthesis were seen in erythroblasts during the first days of culture However, the nearly pure erythroblastic population of the primary inoculum changed to vigorously growing granulocytic cells and macrophages during the 8 day assay period It seems likely that end product inhibition is operative within the monocyte macrophage and granulocyte cell lineages respectively, when cells grow in diffusion chambers *in vivo*

It has been shown that intraperitoneal diffusion chambers provide a good assay system for the study of normal and malignant haematopoiesis [2, 7, 12, 14, 16] The diffusion chamber technique seems to be particularly well suited for the quantitative evaluation of growth of haematopoietic cells [1] Murine haematopoietic stem cells proliferate and differentiate well in these closed *in vivo* cultures, granulocytes and macrophages usually dominate the cultures, whereas the occurrence of other cell lineages is more or less occasional [for a review, see BENESTAD and BREIVIK, 3]

It has been concluded that the diffusion chamber milieu does not facilitate the survival of bone marrow erythroid cells [1] Since fetal erythroid cells probably have a better growth potential, we chose liver haematopoietic cells of 14- to 16-day-old mouse fetuses for proliferation studies in diffusion chambers We here report how the initially nearly pure erythroblastic population changes to vigorously growing granulocytic and macrophagic cells during the 8-day assay period

## Material and Methods

Mice of a random bred NMRI strain were used throughout the study. Diffusion chamber recipients were 6- to 10-week-old virgin females. Fetal mice of 14- to 16-day gestation served as cell donors. In two of the three culture series the chamber recipient mice were given 700 r of whole body irradiation 1 day prior to chamber implantation. The irradiation was produced by a 250 kV X ray apparatus. The physical conditions were: current 10 mA, 1.5 mm Cu filter, target distance 50 cm, and dose rate 85 r/min. The irradiation was omitted in the third culture series because many of the young mice did not survive the whole 8-day assay period after this treatment.

Fetal livers were removed surgically, cut into 2-3 mm pieces, and aspirated through progressively smaller needles (1.4-1.0-0.8-0.6-0.5 mm Ø). This resulted in a single cell suspension. The suspension medium was RPMI 1640 (Orion Pharmaceutical, Helsinki), containing 10% heat inactivated fetal calf serum (Flow Laboratories, Rockville, Md.). In the primary cell suspension about 95% of the nucleated cells were erythroblasts, and 2-4% granulocytic cells. Additionally, there were macrophages/monocytes, megakaryocytes, blasts, and some unidentified cells. A few liver parenchymal cells were also present.

Diffusion chambers with a capacity of about 130 μl were prepared of acrylic rings and Millipore filters as described earlier [15]. The chambers were filled with 100 μl of single cell suspension containing $5 \times 10^5$ nucleated cells. Two chambers glued together were implanted into the abdominal cavity of each recipient mouse. The mice were killed by neck luxation after 1, 2, 3, 5, and 8 days of culture. The chambers were cleaned with lens paper and then treated unopened in 0.5% Pronase solution (Pronase, B grade, Calbiochem, Los Angeles, Calif.) for 70 min. Cells were aspirated from the chambers and the inside of the chambers was washed five times with saline.

Cell counts were made haemocytometrically using acetic acid dilution to a final concentration of 1%. At least 100 nucleated cells were counted. Differential counts were made from cytocentrifuge preparations (Shandon Scientific, London) after staining with the May Grünwald-Giemsa technique. About 500 cells were counted for the differentials. Haematopoietic cells were classified in the following categories: (1) nucleated erythroid cells, (2) granulocytic cells, (3) macrophages (monocytes included), and (4) others.

## Results

In cell numbers between cultures carried by pre-irradiated and untreated mice, statistically significant differences could not be demonstrated. Hence, results from the three culture series were combined. The total cell number did not change much during the whole 8-day assay period (fig. 1). The proportion of cells classified as 'others' (mainly blasts, but also megakaryocytes etc.) never exceeded 5% of the total harvest. Considerable differences were seen in the survival and growth of different cell types.

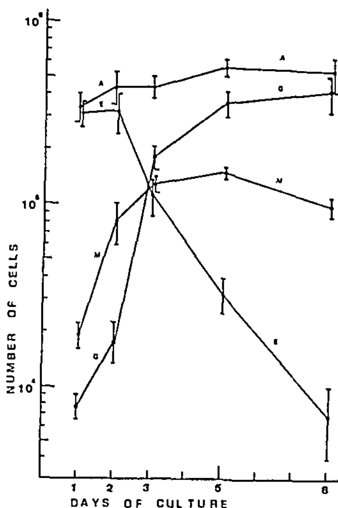



*Fig 1* Growth of haematopoietic cells of mouse fetal liver in diffusion chambers Culture periods were 1, 2, 3, 5, and 8 days Primary inoculum was $5 \times 10^5$ nucleated cells. Values are mean (±SE) of 10 chambers (of 8 chambers on day 8) A = All cells; E = erythroblasts; G = granulocytic cells, M = macrophages (monocytes included)

The number of erythroblasts decreased with time (fig. 1). However, mitotic figures were seen in erythroid cells even on the third day of culture. The haemoglobin content of some erythroblasts increased during the first 2 or 3 days. It is also possible that nuclear ejection of normoblasts took place in the cultures; there were numerous small nuclei free and also phagocytized in the cytoplasm of macrophages. The number of granulocy-

tic cells increased strongly between days 1 and 3 (fig 1) The population doubling time for all granulocytic cells was about 10 h between days 1 and 3, and even shorter between days 2 and 3, being about 7 h In the beginning the majority of granulocytes were young proliferating cells, but the degree of maturation increased considerably during the culture The growth rate of granulocytic cells decreased after 3 days The net growth was nearly zero between days 5 and 8 From days 1 to 8 the total number of granulocytic cells had increased about fiftyfold. The number of cells classified as macrophages increased rapidly between days 1 and 3 (fig 1) Thereafter, the growth rate slowed down. After 5 days of culture the net growth became negative However, mitotic figures were still seen The percentage of other haematopoietic cells (group 4) did not markedly alter with time

## *Discussion*

In the present work a new target cell population, namely fetal liver erythroblasts, was cultured It was not possible to identify all cell types reliably in the chamber cultures, in our opinion, classification of cells is indeed impossible on morphological grounds only However, this does not seriously invalidate the differential counts of the present work, because the great majority of cells (95% or more) belonged to the erythroid granulocytic, and macrophage series, which generally are easy to recognize Although the number of erythroid cells decreased during the culture, active erythropoiesis could be demonstrated during the first 3 days Hence, it is possible that the small positive net growth seen in the growth curve of erythroid cells between days 1 and 2 is real In addition to cell proliferation, haemoglobin synthesis was apparent. Despite an overall negative net growth of erythroid cells in chamber cultures fetal liver cell populations seem to be suitable for studying haemoglobin synthesis as well as erythropoiesis In fact, we have shown that subcutaneous erythropoietin injections into chamber bearing mice markedly increase the incorporation of $^{59}Fe$ into chamber cells although the basal rate of $^{59}Fe$ uptake is already high [unpublished results] The rapid proliferation of granulocytic cells ($T_{d,gr} \sim 7$ h) observed in this work is not unique, similar results have been presented earlier [6] The growth rate of granulocytic cells slowed down after 3 days of culture At the same time the majority of granulocytic cells began to be more mature, post mitotic cells, degenerated cell forms were also common in the cultures of longest duration.

The following conclusions concerning the control of granulopoiesis in the present assay system seem to be justified

(1) Granulopoiesis was not inhibited at the time when the number and the rate of loss of erythroid cells was highest In other words, erythroid cells or their degradation products do not affect granulopoiesis in a non-specific manner

(2) LAERUM *et al* [9] have shown that diffusion conditions through the membranes of the chambers are good for about 3–5 days Furthermore, the exponential growth of many kinds of cell lineages usually takes place in diffusion chamber cultures during the first week Hence, it seems likely that the decreased growth rate of granulocytic cells after 3 days as seen here was not caused by impaired nutritional conditions or accumulation of toxic substances inside the chambers

(3) It has been speculated [13] that an end product stimulation may play a role in the specific regulation of granulopoiesis In the present system a considerable part of granulocytic cells in older cultures were mature and partially degenerated However, any stimulation of granulopoiesis could not be detected

(4) The colony-stimulating factor, evidently produced by monocytes-macrophages [8, 11], may have a central role in the steering of granulopoiesis In the present culture system, the number of cells of the monocyte macrophage series was high between days 3 and 5, but no stimulation in the growth of the granulocytic cells could be noted Thus, a possibly colony stimulating factor originating inside the diffusion chambers does not seem to play a role in the steering of granulopoiesis

(5) Injection of the granulocyte chalone into mice carrying diffusion chambers inhibits DNA synthesis in proliferating granulocytes in the chamber cultures [4, 10] The inhibition of cell growth after 3 days of culture in the present system may also be explicable in terms of a feedback inhibition inside the granulocyte system Of course, the loss of granulocytes increases with time because there are more mature cells in older cultures

End product inhibition might also be operative within the monocyte-macrophage cell lineage when the net growth diminished since day 3 This has also been suggested by others [5, 6]

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# The Fibrinolytic Enzyme System in Anorexia Nervosa

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*Key Words* Anorexia nervosa Fibrinogen Fibrinolysis Plasminogen activation

*Abstract* The levels of components of the fibrinolytic enzyme system in 7 patients with anorexia nervosa were compared with those of healthy young women Plasminogen activator levels were significantly higher and the mean fibrinogen concentration was lower in the anorexia nervosa patients

A number of studies have demonstrated that plasma fibrinolytic activity is impaired in obese persons [5, 13, 16] It has been reported that an increase in fibrinolytic activity occurs during weight reduction in the obese [4, 13], and that short term negative caloric balance also causes the fibrinolytic activity to increase in persons of normal body build [13] We report a study on the fibrinolytic enzyme system in patients with anorexia nervosa to determine whether chronic negative caloric balance is associated with a sustained increase in fibrinolytic activity and whether it results in alterations in the other components of the fibrinolytic system

## *Methods*

*Plasminogen activator* was assayed by performing euglobulin clot lysis times by the method of NILSSON and OLOW [11] using a clot lysis time recorder (Carmanan Instrumentation, Glasgow) The results were expressed by plotting the times logarithmically against units of fibrinolytic activity [17] 10 U being arbitrarily equated with a lysis time of 50 min

*Plasminogen* was measured by the method of ALKJAERSIG *et al* [1] and expressed in Sherry units *Fibrin degradation products* (FR antigen FDP) *in serum* were assayed by the tanned red cell haemagglutination inhibition technique of MERSKEY *et al* [10]

*Table I* Components of the fibrinolytic enzyme in control subjects and patients with anorexia nervosa

| Subject No | Age years | Height cm | Weight kg | O/S ratio | Plasminogen activator U | Plasminogen (casein) U/ml | Fibrinogen mg/dl | FDP μg/ml | $\alpha_1$-Antitrypsin mg/dl | $\alpha_2$-Macroglobulin mg/dl |
|---|---|---|---|---|---|---|---|---|---|---|
| *Controls*[1] *(n = 10)* | | | | | | | | | | |
| | 20.9 ±0.6 | 163.1 ±5.5 | 58.5 ±6.1 | 106 ±10 | 2.7 ±1.9 | 3.7 ±0.5 | 365 ±42 | 1.7 ±1.4 | 349 ±99 | 298 ±98 |
| *Anorexia nervosa patients* | | | | | | | | | | |
| 1 | 19 | 167.6 | 38.1 | 66 | 7.8 | 4.2 | 300 | 0.3 | – | – |
| 2 | 20 | 160.0 | 35.0 | 65 | 7.2 | 4.3 | 310 | 5.1 | 300 | 376 |
| 3 | 23 | 167.6 | 39.0 | 68 | 4.7 | 4.6 | 300 | 6.8 | 368 | 312 |
| 4 | 16 | 166.4 | 43.1 | 75 | 5.0 | 3.9 | 300 | 2.9 | 255 | 254 |
| 5 | 35 | 157.5 | 31.8 | 62 | 6.0 | 3.4 | – | – | – | – |
| 6 | 17 | 162.6 | 31.8 | 58 | 9.2 | 3.2 | 275 | 3.8 | 360 | 396 |
| 7 | 26 | 179.1 | 40.4 | 63 | 7.0 | 3.5 | 250 | 1.2 | 330 | 300 |
| Mean | 23.7 | 165.8 | 37.0 | 65.3 | 6.7 | 3.9 | 289 | 3.4 | 323 | 328 |

[1] Mean ± SD

*Fibrinogen* was measured by a modification [12] of the method of RATNOFF and MENZIE [14]. *$\alpha_2$-Macroglobulin and $\alpha_1$-antitrypsin* levels were assayed by radial immunodiffusion [8] using reagents obtained from Behringwerke AG

*Patients* Seven patients with anorexia nervosa were studied. All were female and had lost weight through active refusal to eat. Amenorrhoea had been present at least 6 months, and in no patient were there features of schizophrenia, severe depression or organic disease. There was no evidence of hepatic dysfunction based on normal values of aspartate transaminase, alkaline phosphatase and serum bilirubin. All were studied before the start of treatment. A further patient who had classical anorexia nervosa was studied following weight regain

*Controls* The control subjects comprised 10 healthy young women aged 20–22 years. In all the observed/standard weight ratio fell between 90 and 118

*Assessment of relative adiposity* Height was measured to the nearest 0.25 in (0.6 cm) and weight to the nearest 0.25 lb (0.11 kg). The ratio of observed to standard weight was calculated according to the weight-for-height standards of KEMSLEY *et al.* [7]. This ratio was found to correlate closely with relative adiposity as estimated by body-density measurement. Blood was obtained from both patients and control subjects between 9 and 10 am after a light breakfast. All rested for a minimum of 20 min before venepuncture.

## *Results*

The mean results for the control women and the individual results for the 7 patients with anorexia nervosa are presented in table I. The striking feature of the patients with anorexia nervosa is their high plasminogen activator activity, the mean level is significantly higher than that of the control women ($p<0.001$). A single patient who had regained weight after treatment was studied at the time her observed/standard weight ratio was 92 and found to have an activator level of 2.0 U, close to that of the mean of the control subjects. The mean levels of plasminogen and fibrin degradation products did not differ significantly from those of the normal women. However, the mean fibrinogen concentration of the anorexia nervosa patients was significantly below that of the control subjects ($p<0.005$). The levels of the major antiplasmins of human blood, $\alpha_1$-antitrypsin and $\alpha_2$-macroglobulin, did not differ significantly between the two groups.

## *Discussion*

This study has demonstrated that patients with untreated anorexia nervosa have high plasminogen activator levels. The single patient studied after weight regain had an activator level in the range of the control subjects, *suggesting that the changes in fibrinolytic activity in patients with anorexia* nervosa are reversible and that they represent a response to chronic negative caloric balance similar to that found in healthy individuals subjected to short-term starvation. Notable is the lack of significant change in the levels of plasminogen or FDP in association with the high activator levels. The failure of plasminogen activation and the appearance of free plasmin activity within the circulation is characteristic of physiological increases in activator level [2].

The explanation for the lower fibrinogen concentration in the patients with anorexia nervosa is unknown. In the absence of a reduction in the plasminogen level or a rise in the FDP level, it is unlikely to be the result of intravascular proteolytic activity or coagulation. Fibrinogen production has been reported to be decreased by starvation when the diet is inadequate in proteins [3, 6]. However, evidence of protein deficiency in anorexia nervosa is minimal [15] and it is unlikely, therefore, that the lower fibrinogen levels are secondary to protein deficiency. It is possible that a relative expansion of the plasma volume, known to occur in anorexia nervosa [9], may contribute to the lowered fibrinogen concentration.

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# Combined Congenital Deficiency of Factor V and Factor VIII

## Report of a Further Case with Some Considerations on the Hereditary Transmission of this Disorder[1]

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*Key Words* Bleeding disorders Combined congenital coagulation defects Factor V deficiency Factor VIII deficiency

*Abstract* A patient with combined factor V and factor VIII deficiency is presented The bleeding manifestations were mild The main laboratory feature was a prolonged partial thromboplastin time which was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum, hemophilia A plasma or plasma of another patient with combined factor V and factor VIII deficiency TGT was also clearly abnormal and was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum Prothrombin consumption was mildly defective The prothrombin time was slightly prolonged Factor VIII was 12% and factor V 55% of normal Factor-VIII associated antigen was normal The father and a sister of the propositus revealed mild factor V deficiency but normal factor VIII activity and antigen The parents were not consanguineous A tentative classification of combined deficiency of factors V and VIII in two groups is proposed The hereditary transmission of the two types of deficiencies is discussed

Combined deficiency of factors V and VIII is a rare coagulation disorder Only 21 cases have been so far described in the literature [1, 10, 14–16, 18–21, 23–25]

The object of the present paper is to present another case with this peculiar defect This is the 22nd case reported in the literature and the 4th in Italy The other cases studied in Italy were reported a few years ago [10, 14] Furthermore, this is the 3rd case for which a normal factor VIII antigen levels is recorded immunologically

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## Case Report

The patient, a 29 year-old male, has been sent to us in February 1975 for an evaluation of his life long coagulation defect. He had been followed by one of us (G G) for the past 3 years. Family history was negative for bleeding disorders but a sister had undergone splenectomy for idiopathic thrombocytopenia. The parents were not consanguineous and came from different regions of Italy The family pedigree is shown in figure 1 During infancy and childhood the patient presented no important bleeding manifestations. He had only easy bruising and occasional epistaxis

At the age of 21, the patient started noticing occasional bleeding from the gums. At that time he was working in a printing company and appeared to be exposed to lead A few months later, the bleeding was attributed to a mild thrombocytopenia even though slightly prolonged glass-clotting and recalcification times had been noted A few months later, platelets were reported to be back to normal At the age of 22, the patient presented excessive bleeding after two tooth extractions. On one occasion he had to be transfused with one unit of fresh blood.

At the age of 26, the patient presented a large hematoma of the left thigh after a minor trauma. He was admitted to a hospital in Genova where, because of leg varices, he was suspected to have a deep thrombophlebitis However, a venographic study showed no deep vein thrombosis The patient was treated with bed rest and the hematoma slowly subsided.

Altogether, bleeding manifestations have not been severe During the last few years a tooth extraction, on two occasions, was followed only by moderate bleeding At the time of study in 1975 there were no bleeding manifestations. All routine laboratory tests, including liver function studies were within normal limits. Respiratory function studies revealed an obstructive pattern compatible with mild asthma.

## Material and Methods

Material and methods have been dealt with in detail elsewhere [5-9] Only new data will be given.

Anti factor VIII inhibitor activity was evaluated according to a modification of the method of BIGGS and BIDWELL [2] A lyophilized factor VIII concentrate preparation (Kryobulin 250, Immuno, Wien) was reconstituted with 20 ml of distilled water according to the manufacturer's instructions. 0.1 ml of this reconstituted factor VIII concentrate preparation was diluted 3-fold with normal saline 0.1 ml of this preparation was then added to 0.9 ml of patient plasma or 'fake' plasma. The fake plasma was obtained by mixing 0.15 ml of normal plasma and 0.85 ml of hemophilia A plasma. The factor VIII level of the fake plasma was 15% of normal. After a 60-min incubation period at 30 °C, factor VIII was assayed in a one-stage system in both mixtures.

Factor VIII associated antigen was evaluated according to a previously reported modification of LAURELL's [11] method. Platelet adhesiveness was measured according to SALZMAN's [22] method.

*Table I* Coagulation study in the propositus

| Test | | Result | Normal values |
|---|---|---|---|
| Platelets/µl | | 170,000 | 150,000–350,000 |
| Bleeding time, min | | 4 | 2–5 |
| Clot retraction | | complete after 10 h | complete after 12 h |
| Glass clotting time, min | | 10 | 5–9 |
| Recalcification time, sec | | 260 | 100–180 |
| Partial thromboplastin time, sec | | 69 2 | 35–45 |
| Prothrombin consumption, % | | 70 | >90 |
| Thromboplastin generation test | | 30 sec in 8 min | 19 sec in 6 or 8 min |
| Prothrombin time, sec | | 16 | 13–14 |
| Stypven-cephalin clotting time, sec | | 14 | 10–12 |
| PP test, sec | | 28 8 | 25–29 |
| Factor II, % | | 100 | 85–115 |
| Factor V, % | | 55 | 85–115 |
| Factor VII, % | | 100 | 85–115 |
| Factor VIII, % | | 12 | 60–160 |
| Factor IX, % | | 100 | 85–115 |
| Factor X, % | | 110 | 85–115 |
| Factor XI, % | | 110 | 70–150 |
| Factor XII, % | | 100 | 70–150 |
| Fibrinogen, mg% | | 480 | 250–500 |
| Fibrinolysis, h | | 15 | 10–30 |
| Thromboelastogram. | r, mm | 26 | 10–20 |
| | K | 12 | 6–12 |
| | ma | 54 | 50–66 |
| Thrombin time, sec | | 19 | 18–25 |
| Platelet adhesiveness, % | | 32 | 20–50 |

*Table II* Partial thromboplastin time (PTT) correction studies

| Mixture, equal parts | PTT of mixtures sec | PTT of reference sec |
|---|---|---|
| Propositus plasma | 69 2 | – |
| Propositus plasma + adsorbed normal plasma | 37 5 | – |
| Propositus plasma + normal serum | 82 | – |
| Propositus plasma + hemophilia A plasma | 90 | 110 |
| Propositus plasma + combined factor V and factor VIII deficiency plasma | 76 6 | 72 |

*Table III* Lack of anti factor V and anti factor VIII activities in the patient's plasma on incubation at 37 °C

| | Incubation time min | | | |
|---|---|---|---|---|
| | 0 | 30 | 60 | 120 |
| Factor V level of a 1 2 mixture of patient's plasma and fresh normal plasma, % | 105 | 96 | 86 | 80 |
| Factor V level of a 1 2 mixture of aged normal plasma and fresh normal plasma, % | 58 | 54 | 50 | 46 |
| Kryoglobulin + propositus' plasma | | | 74 | |
| Kryoglobulin + fake plasma | | | 70 | |

*Table IV* Factor V activity, factor VIII activity and factor VIII antigen in the family members of our propositus

| Family of patient | Factor V % | Factor VIII % | Factor VIII antigen |
|---|---|---|---|
| Propositus | 55 | 12 | 150 |
| Mother | 92 | 110 | 140 |
| Father | 60 | 100 | 120 |
| Brother, $II_1$ | 110 | 105 | 120 |
| Sister, $II_2$ | 55 | 110 | 160 |
| Brother, $II_3$ | 105 | 110 | 115 |
| Sister, $II_4$ | 100 | 120 | 90 |
| Brother, $II_5$ | 96 | 100 | 110 |
| Normal | 85-120 | 60-160 | 60-160 |

Number after brothers and sisters of our propositus correspond to those on the genealogic tree on figure 1

## Results

The results of the coagulation study are summarized in table I Routine tests revealed the presence of a first- and second-stage deficit Partial thromboplastin time (PTT) was clearly prolonged and was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum, hemophilia A plasma or plasma of another patient with combined deficiency of factors V and VIII (table II)

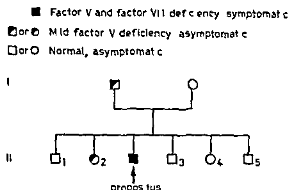



*Fig 1* Family pedigree No paternal relative was available for study

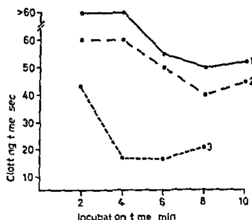



*Fig 2* Thromboplastin generation test Curve 1 refers to the basal curve Curve 2 was obtained after the substitution of patient s serum with normal serum Curve 3 was obtained after the substitution of the patient's adsorbed plasma with adsorbed normal plasma The pattern of curve 3 is normal

The thromboelastogram showed a mild prolongation of r+K, whereas ma was within normal limits Prothrombin consumption was moderately defective The thromboplastin generation test was abnormal too and was corrected by the addition of adsorbed normal plasma (fig 2)

Factor V was 55% and factor VIII was 12% of normal Fibrinogen and factors II, VII, IX, X, XI and XII were all within normal limits Factor-VIII-associated antigen was normal No anti factor or anti-factor VIII inhibitors were present in the propositus plasma (table III) Platelet and vascular tests were within normal limits and there was no hyperfibrinolysis Platelet adhesiveness to glass was within normal limits too

*Table V* Tentative classification of combined deficiency of factors V and VIII

| | Type I, pure association type | Type II, common gene abnormality | Comment |
|---|---|---|---|
| Frequency | rare (1) | common (1) | (1) on the basis of reported families |
| *Consanguinity* | *no* | *frequent* | |
| Factor V level | intermediate (2) | low (3) | (2) compatible with heterozygosis, clearly higher than factor VIII, (3) similar to factor VIII level |
| Factor VIII level | low | low | |
| Hereditary transmission | independent segregation of the two defects | autosomal, recessive | |
| Associated anomalies | no | frequent | |

The father and one sister have mild factor V deficiency (table IV) Factor VIII was normal in all relatives studied both as activity and as antigen In particular, factor-VIII antigen versus factor-VIII activity was practically equal to 1 in all instances

## *Discussion*

The clinical and laboratory features of this disorder appear to be well defined It is a first- and a second-stage defect characterised by a combined deficiency of factors V and VIII The only doubt remaining about the condition concerns its hereditary transmission

A few years ago, we have formulated the possibility that two types of combined deficiency of factors V and VIII may exist [19] The first group would consist of patients in whom a mild factor V defect compatible with heterozygosis is present together with hemophilia A In this case, a hemophiliac could have inherited one abnormal gene controlling factor V synthesis from either of the two parents No consanguinity is present between the parents The second type of defect would consist in an abnormality of one gene or a system of genes controlling a common step in the activation

of both factors V and VIII In this latter case, the condition appears to be transmitted as an autosomal recessive trait and consanguinity is frequently present between the parents One of the patients presented by GOBBI *et al* [14] is probably a typical example of the first type of defect The patients presented by SAITO *et al* [21], JONES *et al* [16], SELIGSOHN *et al* [24] and our previously reported case [10] belong to the second group The first group of patients could be termed type I or pure association type, whereas the second condition could be named type II The distinguishing features are summarized in table V

This tentative classification does not exclude the possibility that other variants of this disorder may exist For example, the family presented by SEIBERT *et al* [23] does not fit the above criteria In this case there was no consanguinity, the mother of the propositus had a factor V level compatible with heterozygosis whereas the factor V level of the propositus was definitely lower and similar to the factor VIII level

The present case, however, seems to confirm our hypothesis since the propositus appears to have a type I disorder Our patient is surely heterozygote for factor V deficiency since other family members have reduced factor V levels and there is no consanguinity between the parents In these cases, an independent segregation of the two defects may occur [14]

The crucial question of this type of combined factor V and VIII deficiency is whether the association is casual or, given a patient with heterozygous parahemophilia, a concomitant factor VIII defect is more likely to occur Parahemophilia is a rare coagulation disorder Only about 60 cases have been found so far in the literature Only 5 surely proven cases have been reported in Italy, together with approximately 20 heterozygotes [4, 7, 8, 16] Hemophilia A is surely a more frequent coagulation disorder About 3,000 hemophiliacs are suspected to be present in Italy Given a population of 55,000,000 that would indicate the presence of 1 hemophiliac in 20,000 The probabilities for a hemophilia A carrier to be also heterozygote for factor V deficiency are indeed very meager but may not be completely ruled out

The mother of our propositus does not seem to be a carrier This interpretation is supported by the factor VIII antigen versus factor VIII activity ratio and by the fact that three additional sons were perfectly normal Therefore, a mutation must be admitted, to explain the presence of a factor VIII defect in the propositus This behavior seems to be the rule in sporadic hemophilia

The existence of a normal factor VIII antigen level in these patients has been demonstrated in both instances in which it has been investigated [12, 26]. The demonstration of a normal antigen level in this third case too, seems to settle definitively the problem. Combined deficiency of factors V and VIII is associated with a normal factor VIII antigen and a low factor VIII activity. This is typical of a hemophilia-like defect. It is interesting to note that in all cases studied, factor VIII antigen level was 100% or greater than 100% of normal. In this regard it may be worth noting that in combined deficiency of factors VII and VIII, a normal factor VIII antigen level was found too [3]. No information, on the contrary, is available for factor V. Is it a real deficiency or a structural defect whereby only activation is abnormal? The question can only be answered when satisfactory antifactor V antiserum is available.

The bleeding manifestations presented by our propositus have not been severe. Hemarthroses has never occurred in our patient but this is not unusual for mild hemophilia patients. In this regard, it may be noted that the factor V level may play an important role in the severity of bleeding. Our previous patient with this disorder had similar factor VIII levels but definitely lower factor V levels (18 versus 55%) than the present case and he had more important bleeding manifestations. The associated presence of leg varices in our propositus is probably only a casual association. The father of our propositus has leg varices too. However, the several associated anomalies such as hypogonadism, mental retardation, psychoneurotic or psychotic traits and enuresis which have been described in patients with this disorder [10, 15, 24]] should be remembered. These associated anomalies have been interpreted as an expression of consanguineous matings. Even in this regard, it may be noted that no associated anomaly has been described in the first group of patients (pure association disease).

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# Brucellosis in Two Thalassaemic Patients Infected by Blood Transfusions from the Same Donor

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*Key Words.* Brucellosis · Thalassaemia · Splenectomy · Blood transfusion

*Abstract.* Brucellosis was diagnosed in two polytransfused, thalassaemic patients who had received blood containing brucella agglutinins from an asymptomatic donor. In one of the patients the onset of the disease simulated infectious mononucleosis. Despite the fact that both cases were splenectomized, the course of illness was not life threatening. However, a tendency for recurrence of the infection and persistence of high brucella agglutinin titres were noted.

Brucellosis is a relatively common disease in many countries. However, its transmission by blood transfusion must be extremely rare since very few cases have been reported in the literature. MENA BRITO [1] mentions 2 cases which were reported at the 2nd Mexican Brucellosis Congress in 1944 and reports 5 more instances of the disease transmitted from donors who had no indication of clinical brucellosis, but had brucella isolated from their blood afterwards. In Mexico, BECERRA-GARCIÁ [2] added 3 cases from a personal communication by CASTANEDA. The only case we could find in the British literature is that of WOOD [3] who described the first case in Great Britain; diagnosis in both the donor and the recipient was based on a suggestive clinical picture and on a very high agglutinin titre, since the blood cultures were negative. There are also few published details on the incubation time, symptoms and clinical course of brucellosis transmitted by blood transfusion.

In this paper we report two cases of brucellosis occurring after transfusion of blood from the same donor who was found afterwards to have had brucella agglutinins in his serum at the time of blood donation. It is of interest that both patients were splenectomized cases of thalassaemia major. In one of the patients the onset of symptoms simulated an infectious mononucleosis like syndrome. These patients attended the out patient Thalassaemia Unit of the Hellenic Red Cross Blood Research Laboratory, for their regular transfusions and haematological follow-up.

## Case Reports

*Case 1.* A 14 year-old girl suffering from homozygous β-thalassaemia had been subjected to splenectomy at the age of 10 for hypersplenism. She had been receiving regular transfusions since the age of 17 months and had had a total of 270 transfusions. On May 5, 1972 she experienced high fever for which she was admitted to hospital. Laboratory examination revealed a positive agglutination test for brucella at a titre of 1:2,500. On the basis of this finding, the diagnosis of brucellosis was made and a course of treatment with tetracycline and streptomycine was given. After an apyrexia of 40 days the patient, was readmitted to hospital for recurrence of symptoms. This time, blood cultures were positive for *Brucella melitensis* and the patient was given cotrimoxazol. After 6 and again after 10 months she experienced two more febrile attacks which were attributed to a relapse of brucellosis and responded to cotrimoxazol and cotrimoxazol plus streptomycine, respectively. The patient was subsequently symptom free but died 1 year later from acute heart failure (table I).

This patient was living in Athens and had had no contacts with cattle, sheep or goats. The possibility of transmission of the disease by blood was considered and the sera of 4 donors who donated blood to her during a 4-month period before the onset of symptoms were examined afterwards for brucella agglutinins. One of the samples belonging to a donor who gave her blood on February 15, 1972 was found positive at a titre of 1:360 while the others were negative.

*Case 2.* A 17 year-old boy suffering also from homozygous β-thalassaemia had his spleen removed for hypersplenism at the age of 16. He was receiving regular transfusions since the age of 2 years and had had a total of 210 blood transfusions. On April 8, 1972 he complained of high fever of a week's duration. Physical examination showed hepatomegaly and enlarged cervical, axillary and inguinal lymph nodes of about 2 cm in diameter. His chest X-ray was normal. White blood cell count was 10,000/µl with metamyelocytes 1%, polymorphonuclears 16%, lymphocytes 40%, monocytes 19%, atypical basophilic lymphocytes 24%. The Paul Bunnell test was negative. The patient became apyretic after 1 week and the lymph node enlargement gradually subsided. On the basis of the haematological findings the clinical diagnosis of infectious mononucleosis was made. 2 months later he was admitted to hospital for high fever accompanied by chills, profuse sweating and prostration. The white blood cell count and differential count were

*Table I* Agglutination titres for brucellosis and clinical course of the donor and the affected patients

| Case | Date | Agglutination titre for brucellosis | Clinical course |
|---|---|---|---|
| Donor | 15 2 1972 | 1 320 | healthy |
| | 29 8 1972 | 1 160 | healthy |
| *Case 1* | *20 4 1972* | *1 2 560* | *onset of fever* |
| | 31 5 1972 | 1 320 | relapse |
| | 15 9 1972 | 1 640 | symptom free |
| | 6 12 1972 | 1 640 | relapse |
| | 7 3 1973 | 1 640 | relapse |
| | 28 9 1973 | 1 320 | symptom free |
| Case 2 | 17 4 1972 | <1 40 | onset of fever |
| | 29 6 1972 | 1 640 | 2nd febrile episode |
| | 10 7 1972 | 1 1,280 | *on treatment* |
| | 29 11 1972 | 1 1,280 | relapse |
| | 17 5 1973 | 1 1 280 | symptom free |
| | 26 9 1973 | 1 320 | symptom free |

normal but a Wright agglutination test was positive to a progressively rising titre (table I) A blood culture was also positive for *Brucella melitensis* A serum sample dating from his first febrile episode was tested and found negative for brucella agglutinins The patient was treated with tetracycline streptomycine and cotrimoxazol for 3 weeks and was soon symptom free 4 months later he had a new febrile attack which was attributed to a relapse of brucellosis and was treated successfully with the same drugs He required no additional treatment and is well 2 years after his last febrile episode

The possibility of transmission of brucellosis to this patient by blood transfusion was again considered since no other source of infection could be implicated An investigation of the source of the transfused blood revealed that on March 23, 1972 the patient had received blood from the same donor with the positive brucella agglutination test as case 1

*The donor* A male farmer aged 26 who had contacts with cattle and goats gave blood for the first time in September 1971 to an unknown recipient On November 1971 he gave blood to a thalassaemic patient who experienced no illness subsequently Then followed two blood donations one on February 2 1972 and another on March 23, 1972 with recipients case 1 and 2 respectively His serum sample of February 2, 1972 was found to contain brucella agglutinins at a titre of 1 320 The donor was contacted again in August 1972 He was well with no abnormal physical signs and insisted that he had never been ill during the previous months or years He had also never been immunized against cholera His serum at that time had a brucella agglutination titre of 1 160

*Discussion*

Brucellosis is endemic in certain areas in Greece and the hazard of transmission of the disease by blood transfusion may be more common than suspected. According to a recent investigation [4] the frequency of positive serum tests in the general healthy population ranges in various areas from 1.7 to 12.8%. In the area of Larissa it was estimated that the frequency of brucellosis was 61.2 cases for 100 000 inhabitants per year. In the Hellenic Red Cross Thalassaemia Unit a total of 5 cases of brucellosis have been recorded during the past 5 years. All of these were thalassaemic polytransfused patients but unequivocal proof of transmission by blood was possible only for the two cases described in this paper. We consider that the high titre of brucella agglutinins in the serum of the donor at the time of blood donation to the two affected patients is highly indicative for active brucellosis. The lack of any clinical symptoms in the donor is not inconsistent with this concept since asymptomatic cases of brucellosis showing occasionally positive blood cultures have been reported [5]. Interesting in that respect are the cases of MENA BRITO [1] to whom brucellosis was transmitted by blood transfusion from asymptomatic donors.

At the University of Minnesota Hospital it was found that the blood of 27 (1.66%) of 1,627 apparently healthy donors whose blood was accepted for transfusion purposes had brucella agglutinins to a titre of 1:160 or higher [5], which is considered a significant diagnostic titre for active brucellosis [6]. It was further shown that representative brucella organisms of the three species remained viable for as long as 6 months when the blood was refrigerated at 4 °C [5]. Yet, no instance of brucellosis transmitted by means of blood transfusion had ever been recognized at the University of Minnesota [6]. According to SPINK [6], except for a possible rare occasion it is doubtful that infection due to *Brucella* abortus can be transmitted by this route. It is however conceivable that infection might be initiated with the more invasive species of *Brucella melitensis*.

It is noteworthy that both our cases were splenectomized patients with thalassaemia major. It is well documented that thalassaemic patients after splenectomy are susceptible to severe and fatal infection from pneumococci, *Haemophilus influenzae* and *Escherichia coli* [7–9]. It appears that the clearance of certain microorganisms such as the pneumococci is defective in these patients [10]. Susceptibility to streptococcal infection has also been reported [11, 12]. It is therefore possible that splenectomized thalassaemic patients may have defective defence mechanisms against brucella trans-

mitted by the intravenous route It is remarkable that in both patients recurrence of the disease occurred despite adequate treatment and a high titre of brucella agglutinins was maintained for a long period after the control of active disease However, the infection was not life-treatening to either patient and responded promptly to antibiotics

Regarding the first pyrexial attack of case 2 which was initially attributed to infectious mononucleosis it is conceivable that this may represent the first febrile attack of brucellosis Lymph node enlargement and a blood picture with lymphocytoid cells similar to those found in infectious mononucleosis have been reported in brucellosis [6, 13–15] The absence of brucella agglutinins at this phase does not exclude this possibility because in brucellosis, several weeks or months may elapse before the appearance of agglutinins which in some rare instances may never develop [6, 16, 17] Because of this it may be difficult in some instances to differentiate brucellosis from infectious mononucleosis acquired by transfusion

Few relevant data exist in the literature concerning the incubation time of the brucella infection transmitted by blood transfusion The incubation time in case 1 was 50 days while in case 2 it could be either 8 days if the first pyrexial attack is to be considered as the onset of brucellosis or 60 days if the second one is considered as such

In this paper we would like to stress the importance of the hazard of brucella infection in polytransfused thalassaemic patients essentially in areas endemic for brucellosis The exclusion of subjects, known to have brucellosis from blood donation, as suggested by WHO [18] will not eliminate the hazard of transmission of the infection

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# Two Serum IgG-M Components of Different Light-Chain Types in a Case of Hodgkin's Disease

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*Key Words* Hodgkin s disease Lymphomas M components Paraproteins

*Abstract* The simultaneous occurrence of two serum and urine M components in a 75 year old woman affected by Hodgkin s disease is reported The patient developed two IgG M components of different light chain types after an episode of pneumococcal pneumonia

Monoclonal gammapathies associated with malignant lymphomas other than Waldenstrom's macroglobulinaemia or heavy-chain disease are infrequent MOORE *et al* [7] studied the electrophoretic patterns of 333 patients with malignant lymphomas and found that 1 5% had monoclonal peaks Comparing these with the incidence of similar monoclonal peaks in normal people, they concluded the occurrence of the IgG peak might be incidental and not related to the lymphoma, whereas the IgM monoclonal peaks occurred much too frequently to be an incidental finding

In some cases of lymphoproliferative disorders, two M components have been reported [2-4] HOBBS [4] and SELIGMANN and BASCH [10] suggested that the appearance of the two M components reflected the presence of unstable, mutating cell populations In Hodgkin's disease and nodular lymphomas, the incidence of monoclonal peaks has been found to be very low [5, 7] The simultaneous occurrence of two serum and urine M components in a case of Hodgkin's disease is reported here

## *Case Report*

A 75 year old woman was admitted to our Division on February, 1972, for neck swelling noted 2 months before We found enlarged lymph nodes only in the right

cervical region while thoracic roentgenograms and lymphography were negative Lymph node biopsy was interpreted as Hodgkin's disease of nodular sclerosing type Laboratory studies including bone marrow examination were normal The Rye classification was used to determine the extent of the disease which was found to be stage I A Between April 11 and 18, the patient received 4,500 mg Natulane i v and an appreciable improvement in cervical adenopathy was noted She was seen again in June 1972 because of thoracic herpes zooster and was given 2.5 g γ-globulins i.v per day as severe hypogammaglobulinaemia was detected

During a bronchopneumonia occurring in the following month the serum protein electrophoresis showed evident changes and in August a single M component was noted In September and November, two distinct M components in the γ region were evident The sternal bone marrow was normocellular erythropoietic and granulocytopoietic lines were normally constituted There was an adequate number of megacaryocytes Plasma cells constituted about 5% of the elements and were not remarkable in morphology, lymphocytes were numerically and morphologically normal

The terminal event was heart failure She died in November 1972 Permission was not granted for autopsy

## *Material and Methods*

Serum protein concentrations were determined by the biuret method Serum electrophoretic studies utilized cellulose acetate as supporting medium [1] and also included immunoelectrophoresis [9] with the use of antisera specific for IgG IgA IgM κ and λ-chains as well as rabbit antiserum against whole normal human serum Immunoglobulins in serum were quantitated by the Mancini method using Partigen from Behringwerke Antisera were purchased from Behringwerke Electrophoresis and immunoelectrophoresis were also performed on dialyzed urine The urine was filtered and then dialyzed against polivinylpirrolidone for 2 days, using dialysis tubing with average pore size 24 Å

## *Results*

Serum protein electrophoresis and values of immunoglobulins are reported in figure 1 A rapid increase of IgG, without significant changes of IgA and IgM was noted in a few months Figure 2 shows the serum immunoelectrophoresis pattern obtained with antiserum against whole normal serum and κ- and λ-chains Figure 3 gives urine protein electrophoresis and figure 4 urine immunoelectrophoresis carried out with IgG antiserum The urinary protein excretion was 300 mg/24 h Bence-Jones protein was associated with global proteinuria

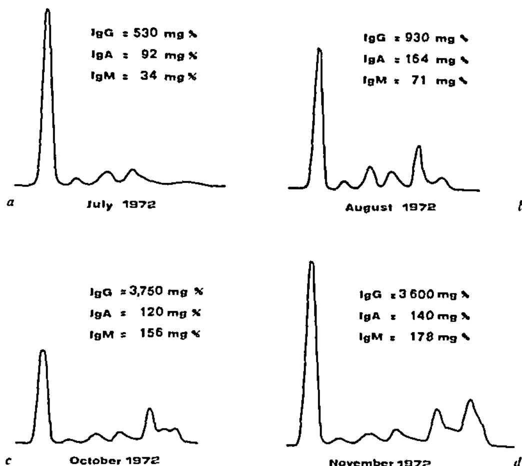



*Fig 1* Serum protein electrophoresis carried out on different occasions *a* Hypogammaglobulinaemia *b*, *c* Single narrow based peaks *c* Two narrow based peaks

## *Discussion*

This case showed the clinical picture of malignant lymphoma which is histologically classified as Hodgkin's disease of the nodular sclerosing type The protein abnormalities were observed during an episode of virus infection complicated with pneumonia and after intravenous administration of γ-globulins Cellulose acetate electrophoresis of serum examined in July showed hypogammaglobulinaemia whereas in August, during the acute phase of infection, a clear single serum M component was noted In the following months two serum M components gradually became evident and the IgG immunoglobulin values presented a rapid increase up to 3 750 mg% while IgA and IgM were within the normal range

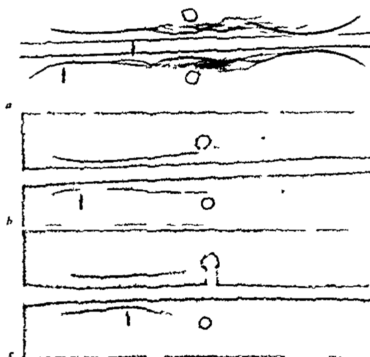



*Fig. 2.* Serum immunoelectrophoretic pattern. Normal serum above the well and patient's serum below the well. *a* Normal human antiserum. *b* *κ Antiserum.* *c* *λ* Antiserum. Arrows indicate the two IgG monoclonal components, the cathodic one of *κ*, the anodic one of *λ* type.

*Serum immunoelectrophoresis* performed with anti-human whole serum in the sample obtained in November confirmed the two M components belonging to IgG immunoglobulins as shown with antiserum with IgG. Using anti *κ* and anti *λ* antisera, it was shown that the two M components were IgG *κ* and IgG *λ*. *Urine immunoelectrophoresis* revealed a light-chain excretion corresponding to the light chain of the serum IgG component.

There was nothing to support the diagnosis of myeloma which rarely has been reported to coexist with classic malignant lymphoreticular disease [6]. The bone marrow did not show myeloma cells *nor* Waldenström's disease tissue.

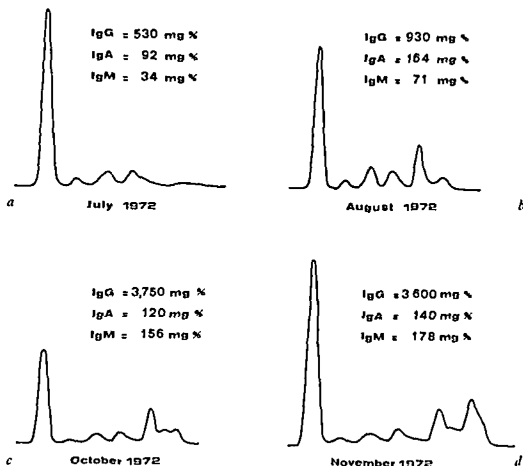



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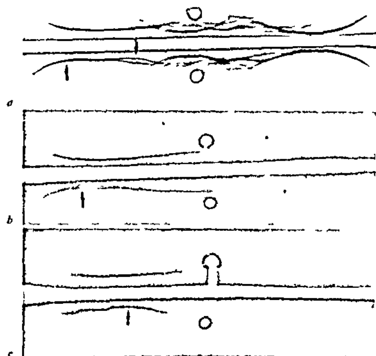



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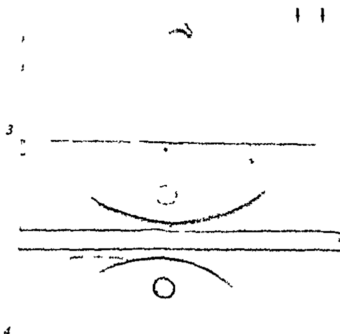


*Fig 3* Electrophoresis of urinary protein concentrate Arrows indicate two narrow bands

*Fig 4* Immunoelectrophoresis on urinary protein concentrate, carried out with IgG antiserum Above normal, below patient s urine

Many investigators support the view that monoclonal protein originates in the neoplastic lymphoid cells [4–6] Others have noted either plasmocytosis or pyroninophilic lymphoid or reticulum cells, or both, in lymph nodes and bone marrow specimens from patients with lymphomas and have speculated that these, rather than the lymphoma, are the source of the abnormal globulin [11, 12] In addition, there are reports of patients with chronic lymphocytic leukemia who developed IgG-M components after an episode of pneumococcal pneumonia [3] or during a hypersensitivity reaction to drugs [8] These cases, as well as our case, presented a profound state of immunological deficiency and they could respond to immunological challenges with monoclonal or biclonal proliferation of immunoglobulin-producing cells

*Acknowledgement* The authors thank Dr F Dammacco for his helpful suggestions

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## Deutsche Gesellschaft für Hämatologie

Die 19 Tagung der Deutschen Gesellschaft fur Hamatologie findet in Freiburg/Breisgau vom 10 bis 13 Oktober 1976 statt Kongress Prasident Prof Dr G W LOHR, Freiburg

Themen Eisenstoffwechsel Arzneimittelbedingte Anamien

Auskunft Priv Doz Dr H ARNOLD, Medizinische Universitatsklinik, Hugstetterstrasse 55, *D-78 Freiburg/Breisgau* (BRD)

## Pappenheim-Preis 1975

Anlasslich der gemeinsamen Tagung der Deutschen und Österreichischen Gesellschaft fur Hamatologie in Bad Nauheim wurde der Pappenheim Preis 1975 am 29 September 1975 Herrn Priv Doz. Dr med RUPERT ENGELHARDT (Medizinische Universitatsklinik Freiburg/Breisgau) fur die Arbeit «Zum Aggregationsverhalten von Proteinen der Erythrozyten Membran» verliehen

## Pappenheim-Preis 1976

Die Deutsche Gesellschaft fur Hamatologie schreibt fur 1976 erneut den Pappenheim Preis aus Der Preis in diesem Jahre mit DM 3000 – dotiert, ist eine Auszeichnung fur eine hervorragende deutschsprachige wissenschaftliche Arbeit, die sich mit klinischen experimentellen oder theoretischen Fragen der Hamatologie befasst Der oder die Autoren sollten nicht alter als 40 Jahre sein Die Manuskripte konnen bis zum 31 5 1976 beim Sekretar der Gesellschaft eingereicht werden Dr med K P HELLRIEGEL, Medizinische Universitatsklinik Joseph Stelzmann Strasse 9, *D-5000 Koln 41* Die Preisverleihung erfolgt wahrend der Eroffnungssitzung der nachsten Tagung der Deutschen Gesellschaft fur Hamatologie, die vom 10 bis 13 Oktober 1976 in Freiburg/Breisgau abgehalten wird

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# Traitement des stades cliniques I et II de maladie de Hodgkin

**Résultats obtenus chez 100 malades par l'association à la radiothérapie d'un ou deux cycles de chimiothérapie**

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*Key Words* Combined chemotherapy radiotherapy of Hodgkin's disease Hodgkin's disease Lymphogranulomatosis

*Abstract* The results obtained by chemo + radiotherapy in 100 patients with Hodgkin's disease at stage I or II as defined by clinical and radiological staging are analysed All patients were treated by chemotherapy followed by radiotherapy, 74 received a complement chemotherapy after the radiotherapy Statistical analysis emphasizes the usefulness of associating two cycles of chemotherapy with the classical radiotherapy With this treatment which gives a constant remission rate of about 90%, a long term chemotherapy is no longer necessary and the interest of exploratory laparotomy becomes negligible

La valeur d'une radiothérapie pour guerir les maladies de Hodgkin apparemment localisées a été bien établie il y a 10 ans lors du Symposium de Paris [9] et n'est pas discutée Nous l'avons appliquée regulierement dès 1965 en obtenant les memes resultats que d'autres équipes Cependant, nous avons été conduits assez rapidement à reconnaitre ses insuffisances, à analyser ses échecs concernant notamment les formes a déplétion lymphoïde [6] et dans ces conditions à lui ajouter un supplément de traitement sous forme d'une association chimiothérapique Dès 1967, nous avons soumis les malades presentant des éléments pronostiques péjoratifs (presence de signes généraux et en particulier, de fièvre [3] type histologique defavorable, tumeurs volumineuses notamment) à un ou deux cycles de chimiothérapie en plus de la radiothérapie classique Une analyse preliminaire (non publiee) ayant mis en évidence que les resultats ainsi obtenus étaient meilleurs que ceux enregistrés avec la radiotherapie isolee pour les maladies à pronostic favorable et la to'e-

rance a l ensemble de la séquence thérapeutique paraissant très acceptable, nous avons généralisé ce protocole de traitement pour tous les malades C est un bilan des resultats obtenus chez les 100 premiers malades ainsi traites que nous exposons ici

## *Malades*

De début 1967 a fin 1974 100 malades pour lesquels le diagnostic de maladie de Hodgkin a pu etre porté et vérifié sur un document histologique indiscutable et qui présentaient un stade I ou II (ganglionnaire d un seul coté du diaphragme) après bilan clinique et radiographique ont reçu en plus de la radiothérapie segmentaire une chimiothérapie complémentaire Le bilan préthérapeutique réalisé a éte celui préconisé à l issue du Symposium de RYE [7] Il n a été fait aucune laparotomie exploratrice

Cette série comprend 66 hommes et 34 femmes dont l age varie entre 6 et 77 ans Les principaux caractères des malades et de leur maladie sont indiqués par le tableau I

Au début de la période considérée seuls les malades présentant un ou plusieurs éléments de pronostic péjoratif ont été soumis à ce protocole Puis à partir de 1972 tous les malades présentant un stade I et II l ont reçu sauf 3 2 pour des raisons non médicales et une femme pour laquelle le diagnostic a été porté au sixieme mois de grossesse

Tous ces malades ont été régulièrement suivis aucun n a été perdu de vue Leurs dossiers ont été exploités courant février 1975

## *Traitement*

Ces 100 malades ont tous reçu d une part, la radiothérapie classique et d autre part un cycle de chimiothérapie préalable Cette chimiothérapie dont les resultats ont été présentés par ailleurs [1] associe vinblastine procarbazine cyclophosphamide et méthylprednisolone selon le schéma indiqué sur la fig 1 Les doses unitaires sont fixes sauf chez les malades pesant moins de 40 kg où elles sont réduites de moitié et c est la durée conditionnant la dose totale qui est modulée en fonction de la surface corporelle et de la leucocytose La durée du cycle ne dépasse jamais 21 jours et le traitement est arrêté quand le nombre des leucocytes atteint 2 000/µl Pour 3 malades on a administré non pas un mais deux ou trois cycles de chimiothérapie à un mois d intervalle avant l irradiation en raison de l existence d une tumeur volumineuse qui a paru insuffisamment réduite après un cycle pour pouvoir etre irradiée dans de bonnes conditions

Chez tous les malades la radiothérapie a eté commencée immédiatement après la fin de la chimiothérapie Pour ceux présentant une atteinte médiastinale ou sus claviculaire la radiothérapie recouvrant les territoires sus diaphragmatique en mantelet et lombo aortique a été administrée en un seul temps chez 58 malades, en deux temps chez les 13 autres avec un intervalle libre d un mois Elle a distribué

*Tableau I* Principaux facteurs pronostiques

| | Nombre de malades | | Nombre de malades |
|---|---|---|---|
| Hommes | 66 | Nombre de territoires atteints | |
| Femmes | 34 | 1 | 22 |
| Age, ans | | 2 | 37 |
| 0 19 | 20 | 3 | 23 |
| 20-39 | 48 | 4 | 8 |
| 40-59 | 21 | 5 | 3 |
| 60 79 | 11 | Médiastin | |
| Histologie | | Normal | 39 |
| I à prédominance | | Envahi | 61 |
| lymphoïde | 14 | Extension juxta ganglionnaire | 13 |
| II, à sclérose nodulaire | 38 | Signes de compression | 11 |
| III, à cellularité mixte | 31 | Signes généraux | |
| IV, à déplétion lymphoïde | 14 | Absents | 68 |
| Inclassable | 3 | Présents | 32 |
| Siège | | Diamètre de la plus grande | |
| Sus-diaphragmatique | 89 | masse tumorale cm | |
| Sous-diaphragmatique | 11 | < 5 | 58 |
| | | 5-10 | 32 |
| | | > 10 | 10 |

en 4 semaines une dose de 4 000 rad dans les territoires ganglionnaires macroscopiquement envahis et 3 500 rad dans les territoires adjacents une surimpression de 500 1 000 rad a été réalisée sur un reliquat tumoral chez 5 malades Le territoire lombo-aortique a été irradié chez 82 malades

Un mois après la fin de la radiothérapie, un nouveau bilan a été réalisé pour apprécier la qualité de la rémission En cas de rémission complete les malades ont reçu un nouveau cycle de chimiothérapie identique au précédent dans les limites de leur tolérance 26 malades n ont pas reçu cette chimiothérapie de consolidation, 21 avant que cette attitude ne soit adoptée et généralisée 3 en raison d une leucopénie persistante et 2 en raison de complication intercurrente

Enfin, après cette deuxième série de chimiothérapie tous les malades ont été laissés sans traitement Aucun n a reçu de chimiothérapie d'entretien

Chez 1 malade, qui était porteur d'une importante splenomégalie l ablation d'une rate de 920 g a été réalisée avant la chimiothérapie initiale

## *Tolérance*

Dans l'immédiat la chimiothérapie initiale n a pas empêché la réalisation de la radiothérapie telle qu elle était prevue Cette irradiation a souvent été commencée

C
V
P
P
1 3 5 7 9 11 13 15 17 19 21 jours
Cyclophosphamide ▼ 200 mg    Procarbazine o 50 mg
Vinblastine 10 mg    Methyl prednisolone 20 mg 80 mg 40 mg

*Fig 1* Schéma du protocole d association chimiothérapique

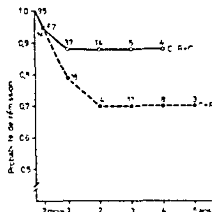
alors que le nombre des leucocytes était de l ordre de 2 000/μl et elle a été pour suivie dans la plupart des cas sans aggraver cette leucopenie Cependant chez 1 malade l irradiation a éte suspendue pendant 15 jours en raison d une leucopénie inferieure à 1 000/μl mais non compliquée avant d etre reprise et menée à terme Par ailleurs 1 malade de 76 ans est décédé dans les jours suivant l achèvement de la radiothérapie dans un tableau de septicémie à staphylocoques que nous avons rattachee au traitement chez les malades qui ont reçu une chimiothérapie de con solidation 4 aplasies medullaires ont été notées à son issue sévères mais reversi bles apres une semaine d hospitalisation supplementaire Sauf pour le sujet décédé aucune de ces complications n a compromis l efficacite du traitement et tous ces malades sont actuellement en rémission persistante En fait loin d avoir compliqué la réalisation de l irradiation la chimiothérapie initiale paraît en avoir amélioré la tolerance grace à la réduction des dimensions des volumes irradiés [2]

*En differe* la responsabilité du traitement (irradiation et/ou association chi miothérapique) a eté seulement suspectée pour une pneumonie fatale non spéci fique verifiée à la necropsie Aucune autre complication n a éte notée Aucun deuxieme cancer n a eté observe A l exception de 2 tous les malades dont la ma ladie de Hodgkin a éte controlée ont repris leur activité professionnelle ou domes tique antérieure

## *Résultats*

Le but initial du traitement est de mettre le malade en *rémission complete* Ce resultat ne peut etre analyse pour 2 malades, l un decédé comme nous l avons vu a l issue du traitement, l autre 15 jours plus tard d hemorragies digestive intercurrentes Pour les 98 malades analysables la remission a ete complete dans 93 cas, incomplete dans 5 cas

Le *maintien de cette rémission complete* est indique par la figure 2 qui separe les malades ayant eu ou non une chimiothérapie de consoli dation il n'y a plus de rechute au dela de 2 ans chez les malades n'ay-



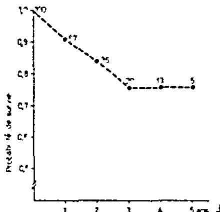


*Fig. 2* Courbes actuarielles des rémissions complètes obtenues après chimio + radiothérapie (C + R) et chimio + radio + chimiothérapie (C + R + C)
*Fig. 3* Courbe actuarielle des survies non corrigées

ant reçu qu'un cycle de chimiothérapie et pour eux l'espérance de guérison se chiffre à 70% et aucune rechute au-delà de 1 an chez ceux ayant reçu deux cycles de chimiothérapie et pour eux l'espérance de guérison atteint près de 90%.

Les 5 rémissions incomplètes à l'issue de la première séquence de chimiothérapie + radiothérapie ont été observées chez 4 hommes et 1 femme de 54, 50, 45, 39 et 20 ans, présentant une forme à prédominance lymphoïde pour l'un d'eux, à cellularité mixte et à sclérose nodulaire pour 2. Ce qui est surtout frappant c'est que 4 d'entre eux présentaient au départ des signes de compression médiastinale nette, pour le 5e la rémission a été jugée incomplète à cause d'une vitesse de sédimentation anormalement élevée qui a été expliquée par une reprise évolutive hépatique ultérieure; ce malade est le seul pour lequel nous ayons pu regretter de ne pas avoir fait une laparotomie exploratrice.

Les 7 rechutes sont survenues chez 4 hommes et 3 femmes présentant une forme à déplétion lymphoïde (3), à sclérose nodulaire (2), à cellularité mixte (1) ou à prédominance lymphoïde (1). Tous, sauf 1, avaient une masse tumorale prédominante mesurant plus de 5 cm de diamètre.

Pour ces 12 échecs, la reprise ou la poursuite évolutive a été située dans les territoires irradiés pour 6 malades, en bordure de champ pour 2 et généralisée pour les 4 derniers.

*La survie brute* portant sur les 100 malades est indiquée par la figure 3. Les 15 décès enregistrés ont été liés à l'évolution hodgkinienne pour 9 d'entre eux, rattachés au traitement pour 1 et à des affections in-

C V P P
1 3 5 7 9 11 13 15 17 19 21 jours
Cyclophosphamide ▼ 200 mg    Procarbazine o 50 mg
Vinblastine 10 mg    Methyl prednisolone 70 mg 30 mg 40 mg

*Fig 1* Schéma du protocole d'association chimiothérapique

alors que le nombre des leucocytes était de l'ordre de 2 000/μl et elle a été poursuivie, dans la plupart des cas, sans aggraver cette leucopénie Cependant chez 1 malade, l'irradiation a été suspendue pendant 15 jours en raison d'une leucopénie inférieure à 1 000/μl mais non compliquée, avant d'être reprise et menée à terme Par ailleurs 1 malade de 76 ans est décédé dans les jours suivant l'achèvement de la radiothérapie dans un tableau de septicémie à staphylocoques que nous avons rattachée au traitement, chez les malades qui ont reçu une chimiothérapie de consolidation, 4 aplasies médullaires ont été notées à son issue sévères mais réversibles après une semaine d'hospitalisation supplémentaire Sauf pour le sujet décédé, aucune de ces complications n'a compromis l'efficacité du traitement et tous ces malades sont actuellement en rémission persistante En fait loin d'avoir compliqué la réalisation de l'irradiation la chimiothérapie initiale paraît en avoir amélioré la tolérance grace à la réduction des dimensions des volumes irradiés [2]

*En différé*, la responsabilité du traitement (irradiation et/ou association chimiothérapique) a été seulement suspectée pour une pneumonie fatale non spécifique, vérifiée à la nécropsie Aucune autre complication n'a été notée Aucun deuxieme cancer n'a été observé A l'exception de 2, tous les malades dont la maladie de Hodgkin a été contrôlée, ont repris leur activité professionnelle ou domestique antérieure

## *Résultats*

Le but initial du traitement est de mettre le malade en *rémission complete* Ce resultat ne peut être analysé pour 2 malades, l'un décédé comme nous l'avons vu a l'issue du traitement, l'autre 15 jours plus tard d'hémorragies digestive intercurrentes Pour les 98 malades analysables, la rémission a ete complete dans 93 cas, incomplète dans 5 cas

Le *maintien de cette rémission complète* est indiqué par la figure 2 qui separe les malades ayant eu ou non une chimiothérapie de consolidation il n'y a plus de rechute au-delà de 2 ans chez les malades n'ay-

par rapport aux résultats obtenus avec la radiothérapie isolée [5] Le bénéfice thérapeutique ainsi enregistré est acquis au prix d'une charge thérapeutique supplementaire initiale mais aussi sans le traitement complémentaire de 2–3 ans par vinblastine souvent preconisé Enfin, ces résultats sont obtenus sans avoir imposé aux malades de laparotomie exploratrice Si cette intervention, qui permet de topographier avec plus de précision que le bilan radioclinique l'extension tumorale, est essentielle quand on réalise une radiotherapie exclusive, elle semble inutile en cas de chimiotherapie associée Celle-ci permet d'atteindre les cellules neoplasiques où qu'elles soient et contribue ainsi de façon décisive, à la guerison des malades

## Résumé

Depuis plusieurs années, nous traitons les malades atteints de maladie de Hodgkin aux stades cliniques I et II par une association radio-chimiothérapique nous présentons ici les résultats obtenus chez les 100 premiers malades ainsi traités Ils ont tous reçu d'abord un cycle de 2-3 semaines de polychimiothérapie d'induction immédiatement suivi de la radiothérapie Trois quarts d'entre eux ont reçu ensuite un cycle de chimiothérapie de consolidation identique au premier Aucun n'a reçu de traitement d'entretien La qualité des résultats et leur stabilité (avec un plateau de rémission de l'ordre de 90% pour les malades ayant reçu deux cycles de chimiothérapie) indiquent que la laparotomie exploratrice comme la chimiothérapie d'entretien ne peuvent apporter dans ces conditions qu'un bénéfice supplémentaire minime leur réalisation est donc inutile si l'on applique un tel protocole de traitement

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# Pathogenetic Aspects of Anemia in Long-Term Hemodialyzed Patients

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*Key Words.* Anemia in hemodialysis · Hemodialysis · Uremia

*Abstract.* The anemia of dialyzed uremic patients is due to several factors: loss of blood and proteins, increased destruction of erythrocytes and alteration of erythropoietin feedback. In an anemic dialyzed patient the number of reticulocytes is normal while a sudden worsening of the anemia is followed by severe reticulocytosis.

Several factors contribute to anemia in a patient undergoing hemodialysis. The loss of blood is considerable: from 1.57 to 4.62 liters/year according to HOCKEN and MARWAH [7], about 10 ml per dialytic session (taking into consideration only the blood residual in the dialyzer) according to BROZOVICH *et al.* [1], from 2 to 4.5 liters/year according to LINDSAY and KENNEDY [16]. The survival of erythrocytes in a dialyzed patient is reduced; the phenomenon is, at least in part, connected with the traumatism caused by the pumps used for the blood flux [11, 13]. Concurring factors may be a deficiency of folate lost through the dialyzer [6] and a deficiency of iron due to both blood loss and impaired absorption [2, 5]. However, KOMINAMI *et al.* [11a] have observed normal serum iron values in a group of dialyzed patients. According to LAWSON *et al.* [14], a deficient erythrocyte incorporation of $^{59}Fe$ exists. Transferrin values are constantly reduced [10, 23]. Finally, the reduction or absence of erythropoietin production appears to be of great importance in the genesis of anemia [5].

In the present paper we refer some personal data which, together with data acquired from other publications, we think can contribute to a better clarification of the problem.

## Material and Methods

In 11 uremic patients undergoing hemodialysis twice a week with Kil s kidney for a total of 22 h (table I) we checked hemoglobin erythrocytes and reticulocytes monthly serum iron once every 2 months transferrin (radial immunodiffusion method) and osmotic erythrocyte resistance every year In add tion we searched for siderocytes burr cells [22] and erythrocyte antibodies (d rect and indirect Coombs test and tests on trypsinized red cells) every year We studied the hemoglobin p c ture at the beginning of dialysis and after 2 years (electrophoresis on cellulose ace tate) We tested the erythrocytes for glucose 6-phosphate dehydrogenase (G 6-PD) [12] and for pyruvate kinase (PK) [1] after about 30 months of dialysis Bone mar row aspiration was carried out after 24 months of dialysis and in case No 2 3 4 5 6 and 8 it was repeated at the 48th 60th 46th 39th 41st and 72nd month of di alysis respectively In addition to the morphological observation we also studied the maturation curve and the mitotic index of the erythroblasts We also did Perls reac tion for iron and PAS staining

We studied erythrocytic morphology with the scanning microscope in 5 patients at the third year of dialysis on samples drawn both at the beginning and at the end of a dialytic session

## Results

Only one of the patients (case No 2 with a polycystic kidney) has an almost normal blood picture, in a second patient (case No 10 suffering from secondary amyloidosis) anemia is very mild, in all other patients the anemia is evident but of varying seriousness The reticulocyte values are practically normal (table I) The hemoglobin electrophoresis gave normal results except in one patient (No 5) who has a trait of thalassemia drepanocytosis The serum iron values are at low normal limits, the trans-ferrin is constantly reduced (table I) The values of erythrocyte G 6 PD are normal or at the low normal limits, PK values are at higher limits or greatly increased (table I) Siderocyte research has always been negative The osmotic erythrocyte resistance is slightly reduced in every patient Burr cells are present in every patient they vary from 7 to 16% in sam ples which we examined immediately, after an hour s incubation period in a damp clamp chamber more than 40% of the erythrocytes present echin ocytic transformation (which on the contrary is not present in test sam ples from normal subjects)

During a first period erythrocyte antibody research was always nega tive in one case (No 7) 31 months after the beginning of dialysis hemo-lytic anemia due to cold anti I antibodies was present The considerable worsening of the anemia caused severe reticulocytosis The regression of

*Table I*

| Case No | Age years | Sex | Months of dialysis | Diagnosis | Transfusions | Hb g% | RBC × 10³/μl | Reticulocytes, ‰ | Serum iron μg% | Transferrin mg% | mU/10⁹ erythrocytes G-6-PD | PK |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 59 | f | 31 | ch. n. | 2[1] | 42 | 2,200 | 11-14 | 80 | 160 | 107 | 423 |
| | | | | | | 45 | 2,400 | | 91 | 160 | | |
| 2 | 52 | f | 48 | p. k. | - | 74 | 4,000 | 7-10 | 81 | 190 | 129 | 267 |
| | | | | | | 80 | 4,500 | | 96 | 210 | | |
| 3 | 43 | m | 60 | ch. p. | 4[1] | 55 | 2,900 | 8-13 | 80 | 170 | 152 | 283 |
| | | | | | | 60 | 3,300 | | 88 | 190 | | |
| 4 | 60 | m | 46 | ch. n. | 4[1] | 55 | 3,000 | 7-14 | 71 | 170 | 146 | 433 |
| | | | | | | 60 | 3,100 | | 84 | 190 | | |
| 5 | 47 | m | 39 | ch. n. | - | 53 | 2,700 | 7-10 | 70 | 160 | 106 | 319 |
| | | | | | | 60 | 3,200 | | 91 | 170 | | |
| 6 | 35 | m | 41 | ch. n. | 6[1] | 40 | 2,200 | 13-16 | 76 | 150 | 120 | 390 |
| | | | | | | 42 | 2,400 | | 94 | 190 | | |
| 7 | 49 | m | 41 | ch. n. | 8 | 38 | 1,800 | 10-15 | 80 | 170 | 111 | 360 |
| | | | | | | 42 | 2,300 | | 98 | 185 | | |
| 8 | 21 | f | 72 | ch. n. | 16 | 18 | 1,900 | 12-17 | 66 | 160 | 174 | 369 |
| | | | | | | 45 | 2,300 | | 79 | 190 | | |
| 9 | 64 | m | 30 | ch. n. | - | 48 | 2,400 | 9-13 | 66 | - | - | - |
| | | | | | | 50 | 2,900 | | 88 | | | |
| 10 | 44 | m | 45 | am. k. | - | 66 | 3,500 | 7-14 | 90 | 155 | 130 | 280 |
| | | | | | | 72 | 4,000 | | 104 | 170 | | |
| 11 | 44 | m | 51 | ch. n. | 18+6[1] | 38 | 1,900 | 8-16 | 75 | 160 | - | - |
| | | | | | | 42 | 2,200 | | 93 | 180 | | |
| | | | | | | | | | normal values | | 120 | 60 |
| | | | | | | | | | | | 240 | 220 |

ch. n. = Chronic nephritis; p. k. = polycystic kidney; ch. p. = chronic pyelonephritis, am. k. = amyloid kidney.

[1] Indicates the transfusions carried out to replace acute blood losses. With regard to case No. 7 the data do not refer to the hyperhemolytic period. Hb, RBC, reticulocytes, serum iron, transferrin: extreme values.



*Fig 1* Erythrocytes in the scanning microscope *a* Tendency to spherocytosis × 1,900 *b* Superficial roughness × 4 900

hyperhemolysis after treatment with azothioprine determined the return to the starting values of both red cells and reticulocytes

The study of the bone marrow showed a normal erythropoiesis without any morphological changes The leucoerythropoietic ratio was increased (equal to 4 2) in patient No 8 and normal in all other patients The maturation curve is normal in all patients except cases No 4 and 8 in whom the basophil phases were prevalent The erythroblastic mitotic index was

normal in cases No 5 and 8, and low in all other cases (between 5 and 8‰) PAS staining gave negative results, the sideroblasts were normal

With the scanning microscope the erythrocytes showed a tendency to spheric deformation, fragmented formes, and shapes with evident superficial roughness (fig. 1) These alterations were identical in test samples drawn both at the beginning and at the end of a dialytic session

## *Discussion*

In the examined patients only case No 2 shows no signs of anemia In all other patients anemia is present though variable In the pathogenesis loss of blood is according to our data [unpublished], a little inferior to that referred in other publications (from 1,600 to 3,000 ml year), it is however, enough to cause a latent iron deficiency We must remember that loss of blood causes a simultaneous loss of proteins with abnormal demands on the protein synthesis [3, 17] The constant hypotransferrinemia in dialyzed patients is probably due to such factors as well as to an insufficient diet

The signs of increased fragility of the erythrocytes could be ascribed to certain uremic poisons which cannot be dialyzed or only with difficulty [4] However, an important factor is the microtraumatism caused by the extracorporeal circulation particularly by the thin strands of fibrin, which constantly form in the dialyzer We must point out that in our patients dialysis was performed with an external arteriovenous shunt without using mechanical pumps for blood flow Mechanical destruction could set off autoimmune phenomena

The red cells of uremic patients do not show any significant metabolic alterations on the contrary an increase of organic phosphates has been observed due to an activation of anaerobic glycolysis which is probably secondary to a rise in plasmatic phosphates [8, 15] Within this ambit the increase of PK values, frequently observed by us can be included

We could not see any megaloblasts or changes of iron utilization in the bone marrow (normal sideroblasts) The erythroblastic mitotic index is reduced in the majority of cases As usual the erythroblasts are PAS-negative However KIRIS and HELLER [7] revealed a high percentage of PAS positive erythroblasts in patients who had died of uremia or were in the terminal stage These contradictory reports may depend on the different seriousness of the disease

In our patients, even in the presence of severe anemia, the number of reticulocytes remained normal, however, in case No 7 further worsening of anemia was followed by acute reticulocytosis An analogous phenomenon was described by SHAW and SHOLES [25] following a severe hemorrhage The observation that the anemia in a dialyzed patient shows a normal reticulocyte count and that reticulocytosis can, however, follow a further critical fall of red cells induces us to suppose a varying change of the erythropoietin feedback mechanism In support is the fact that the kidney is not the only site of erythropoietin production [19, 21] while it certainly induces a normal erythropoietin feedback mechanism [18] On the other hand the increase of 2,3-DPG and ATP of the erythrocytes between the dialyses [15, 23] may shift the oxygen dissociation curve of the hemoglobin rightward, providing an increased amount of oxygen to the tissues so as to 'deceive' the structures that produce erythropoietin

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# Erythroblastic Islands and Extra-Medullary Erythropoiesis in Chronic Myeloid Leukaemia

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*Key Words* Anaemia in leukaemia  Erythroblastic islands  Extramedullary erythropoiesis  Leukaemia

*Abstract* Aspirates from bone marrow, spleen and liver were morphologically analysed in 15 untreated patients with chronic myeloid leukaemia Megaloblastic changes of the erythroblasts were found to be more common in the spleen and liver than in the bone marrow A significant increase of 'erythroblastic islands', i e erythroblasts in contact with reticulum cells, were recorded in the leukaemia patients compared to 15 healthy controls It is suggested that the presence of such formations may indicate an increased death rate of defective erythroblasts followed by phagocytosis into reticuloendothelial cells

The successive development of anaemia and hepatosplenomegaly are regular clinical features in chronic myeloid leukaemia (CML) It has previously been demonstrated that haemolysis [2], haemodilution and spleen sequestration of erythrocytes [3–5] may contribute to the anaemia A reduced differentiation of basophilic erythroblasts towards more mature cells has also been proposed [9, 11] Mitotic and morphologic studies of the spleen and liver erythroblasts have given evidence for a more defective function of the erythropoietic pool in the extramedullary sites than in the bone marrow [9, 12, 13]

Leukaemic involvement of the erythroblasts in erythroleukaemia and acute granulocytic leukaemia has been shown in many investigations with various techniques [for review see 10] It has been suggested that the abundance of erythroblastic islands in acute myeloid leukaemias with a high frequency of megaloblastoid erythroblasts may be signs of ineffective erythropoiesis [10]

The present work was undertaken to investigate whether the morphol-

ogy of the erythropoietic tissue of the spleen and liver might indicate some abnormalities in the extramedullary production of red cells in CML. In addition to this a comparison between the intra- and extramedullary erythropoietic tissue was justified, since previous investigations have indicated that the extramedullary erythropoietic pool forms a considerable contribution of erythroid precursors [2, 8, 9, 12–14]. Special interest was devoted to the abundance of erythroblastic islands in the bone marrow, spleen and liver of the CML patients. It has previously been suggested that the presence of such formations may indicate phagocytosis of erythroblasts into reticulum cells [9, 10], thus being a sign of increased death rate of abnormal erythroblasts.

## *Material and Methods*

*Patients.* 15 untreated patients with a recent diagnosis of CML were examined. There were 10 men and 5 women with a median age of 51 years. No patient was in blast crisis. The WBC counts were 76 000–311 000.

*Controls.* 15 apparently healthy persons, 7 men and 8 women, with normal haematological data and normal erythrocyte sedimentation rate served as controls. Their age was 19–82 years (median 51).

*Morphologic investigations.* Bone marrow was obtained through sternal puncture and material from the liver and spleen through fine-needle aspiration biopsy as described by SÖDERSTRÖM [14]. Aspirates from the three organs were obtained on the same occasion and smears were stained with May-Grünwald-Giemsa. In each smear 1 000 erythroblasts were examined and morphologically classified according to HEILMEYER and BEGEMANN [7] and SJÖGREN [9]. Proerythroblasts and basophilic erythroblasts were then pooled into one group and denominated basophilic erythroblasts. The proportion of erythroblasts with a megaloblastic morphology was determined. When erythroblasts were found in contact with or apparently lying within a reticulum cell the formation was classified as an erythroblastic island, and the number of such formations per 1 000 erythroblasts was registered. It proved impossible to carry out a blind analysis of the smears from the bone marrow, spleen and liver, since the splenic pulp cells and the liver parenchyma cells revealed the origin of the smears.

*Statistics.* The results are expressed as median values and ranges. The Wilcoxon matched pairs signed ranks test and the Mann Whitney U test were used to assert significance of the results.

## *Results*

The proportion of basophilic erythroblasts was on average 37.8% (24.5–53.8) in the bone marrow, 38.4% (21.8–53.4) in the spleen and 40.0% (23.0–61.6) in the liver. The differences are not significant.

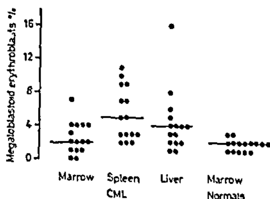



*Fig 1* Percentage of erythroblasts with megaloblastic changes in the bone mar row, spleen and liver of 15 CML patients and in the bone marrow of 15 healthy controls Median values are indicated

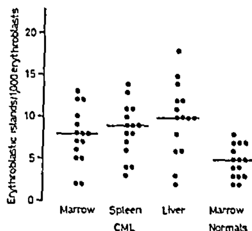



*Fig 2* Frequency of erythroblastic islands in the bone marrow, spleen and liver of 15 CML patients and in the bone marrow of 15 healthy controls

The proportion of megaloblastoid erythroblasts was 2 1% in the bone marrow of the CML patients compared to 5 3% in the spleen The difference is significant ($p<0 02$) In the liver there were 3 8% megaloblasts This proportion is significantly higher than in the bone marrow ($p<0 05$) In the controls there were 1 7% megaloblasts in the bone marrow There is no significant difference between the bone marrow proportions of CML patients and the controls (fig 1)

The number of erythroblastic islands was 8/1,000 erythroblasts in the bone marrow, 9/1,000 in the spleen and 10/1,000 in the liver of the CML patients In the controls there were 5/1,000 erythroblasts in the bone

marrow The difference between the CML patients and the controls is significant ($p<0.05$, fig. 2)

In the CML patients the number of erythroblastic islands was correlated to the percentage of basophilic erythroblasts within the erythropoietic pool, $r_s = 0.86$, $p<0.001$ in the bone marrow, $r_s = 0.82$, $p<0.01$ in the spleen, and $r_s = 0.94$, $p<0.001$ in the liver (the Spearman rank correlation coefficient)

## *Discussion*

The present data indicate that the extramedullary erythropoiesis in CML obviously fails to compensate factors that promote the development of anaemia Although previous investigations [8, 9, 12, 13] have demonstrated that there are higher proportions of erythroblasts in the extramedullary haemopoietic tissue than in the bone marrow, these erythroblasts have low mitotic indices indicating an impaired proliferative activity [9, 12, 13] *The composition of the erythropoietic pools of the intra and extramedullary* organs was probably the same with regard to erythroblasts at a different stage of maturation, since the proportions of basophilic erythroblasts were found to be the same in the bone marrow, spleen and liver It has previously been proposed that the differentiation of early erythroblasts towards more mature erythroid cells becomes progressively blocked during the course of the disease, resulting in an accumulation of basophilic erythroblasts in the bone marrow of the CML patients [9, 11] The present investigation gives evidence for a maturation arrest of the extramedullary erythroblasts as well as the erythroid cells of the bone marrow However, megaloblastic changes, indicating a disturbed DNA synthesis, were more common in the extramedullary organs which is in agreement with previous observations [9, 12, 14] Megaloblasts and disturbed DNA synthesis have also been reported in other morphological and biochemical investigations on CML [1, 6] The mechanisms causing an impaired extramedullary production of erythroblasts are obscure. The discrepancies observed may be due to environmental differences between the bone marrow and the extramedullary organs.

Using various techniques some investigators have proposed that phagocytic reticuloendothelial cells may devour abnormal erythroblasts [for review see 9, 10] The increased frequency of erythroblastic islands is probably the morphological sign of haemolysis. The abundance of erythroblastic islands in the extramedullary organs is in agreement with find-

ings of a latent haemolytic syndrome of the splenomegalic CML patients [2] The observations of an abnormally large porportion of basophilic erythroblasts and its correlation to the number of erythroblastic islands are compatible with an impaired differentiation towards more mature erythroid precursors An ineffective erythropoiesis with an increased death rate of erythroblasts within the haemopoietic tissue may, therefore, contribute to anaemia in CML as well as in the acute myeloid leukaemias

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# Oxymetholone Effect on Acute Myeloblastic Leukemia Cells *in vitro*[1]

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*Key Words.* Androgens · DNA synthesis · Leukemia · Oxymetholone

*Abstract.* The possibility that oxymetholone might induce or enhance leukemia after androgen therapy for aplastic anemia prompted us to study the direct action of oxymetholone on the DNA synthesis of AML cells *in vitro*. The peripheral blasts of 10 patients, 8 with AML and 2 with CML in blast crisis have been studied. The DNA synthesis of the leukemic cells with and without oxymetholone was measured by the ³H methyl thymidine incorporation determined by liquid scintillation. The results have shown a wide variation of DNA synthesis from patient to patient with a range from 2 000 to 40 000 cpm but no significant difference between test and control cultures. We may conclude that oxymetholone does not increase directly the proliferation capacity of the peripheral AML cells cultured *in vitro*.

The paper by DELAMORE and GEARY [5], reporting four patients who developed acute myeloblastic leukemia after treatment of aplastic anemia with oxymetholone, prompted us to study the direct action of oxymetholone on the DNA synthesis of acute myeloblastic leukemia (AML) cells *in vitro*.

## *Materials and Methods*

Ten patients, eight with AML and two with CML in blastic crisis have been studied. The diagnosis of AML was based on the clinical findings and the cytological abnormalities observed in the peripheral blood smear and the bone marrow. All eight cases of AML were confirmed by cytochemical reactions. There was no case of promyelocytic or monoblastic leukemia, although patient 6 could be cytologically

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but not cytochemically considered as a myelomonocytic leukemia. None of the eight patients with AML had been previously treated, thus they all were in their first perceptive phase of the disease

The two cases of CML in blastic crisis had both previously received Busulfan and their diagnosis had been established 2 and 4½ years before their first signs of blastic crisis In all cases, peripheral blast cells were higher than 80% except for the two cases of blastic crisis that were 58 and 35%

5 ml of venous blood were withdrawn into a sterile tube containing 0.5 mg heparin Cells were let to sediment at 37 °C for about half an hour, and the leukocyte layer was withdrawn Cultures were prepared with a final peripheral blast concentration of $10^6$/ml Culture medium 199 TC Difco was supplemented with 20% AB serum

Aqueous suspension of oxymetholone (provided by Syntex) at a final concentration of $3 \times 10^{-6}$ M was added to the test cultures Test and control cultures were assayed by addition of $^3$H methyl thymidine 1 μCi/ml (spec act 1 9 Ci/mM) at the beginning of the 4 hour cultures All cultures were done in duplicates DNA synthesis was measured according to DUPONT *et al* [6] as cpm/$10^6$ cells in a Beckman LS 250 liquid scintillation system

## *Results*

The results have shown a wide variation of DNA synthesis from patient to patient with a range from 2,000 to nearly 40,000 cpm (table I) There is a tendency to lower values when the peripheral blast count is higher, but this is not true in all cases The lowest value was found in the cytologically considered acute myelomonocytic leukemia (case 6), but there was also a low value in cases 4 and 8 where we would expect a much higher thymidine incorporation The two patients with CML and blast crisis showed also divergent results with a very low incorporation in case 10 in spite of a reasonable number of blast cells and a very aggressive disease

We could not find any correlation between cpm and response to treatment Cases 1, 2, and 7 showed complete remission, case 4 had a partial remission and cases 3, 5, 6 and 8 showed no response to therapy Thus, cases with high and low thymidine incorporation showed no response, but the cases which responded had a tendency to higher values Both cases with CML and blastic crisis died without response

Our study shows no statistically significant differences between control and test cultures with oxymetholone In some cases, we even noted a slight decrease of thymidine incorporation Therefore, we may conclude that oxymetholone does not increase directly the proliferation capacity of the peripheral AML cells cultured *in vitro*

*Table I* Results of ³H thymidine incorporation by peripheral blast cells cultured *in vitro* with and without oxymetholone

| Case | Sex | Age years | Type | Peripheral blast count | Control cpm/$10^6$ cells | Oxymetholone cpm/$10^6$ cells |
|---|---|---|---|---|---|---|
| 1 | M | 22 | AML | 86 000 | 20,368 | 19 957 |
| | | | | | 19 866 | 17 603 |
| 2 | M | 18 | AML | 33 000 | 27,831 | 28,335 |
| | | | | | 29,254 | 26 941 |
| 3 | F | 46 | AML | 142,000 | 3,310 | 2,858 |
| | | | | | 3 913 | 3 709 |
| 4 | M | 65 | AML | 18 000 | 12,576 | 13 467 |
| | | | | | 10 895 | 12,381 |
| 5 | M | 38 | AML | 21,500 | 39,232 | 37 628 |
| | | | | | 36,779 | 38 922 |
| 6 | M | 59 | AML | 42,000 | 2,059 | 1 935 |
| | | | | | 2,372 | 2,151 |
| 7 | F | 34 | AML | 97 000 | 18 744 | 16,582 |
| | | | | | 17 657 | 16,938 |
| 8 | M | 37 | AML | 26,500 | 7,241 | 7,952 |
| | | | | | 7 833 | 6,505 |
| 9 | F | 43 | CML | 29 000 | 22,079 | 23 670 |
| | | | BC | | 24,286 | 21 432 |
| 10 | F | 61 | CML | 38 500 | 5 657 | 4,350 |
| | | | BC | | 4 118 | 5,221 |

## *Discussion*

We are aware of the limited values of these results as we have been only working with peripheral blast cells. It might be possible that the bone marrow blast cells would respond to oxymetholone, but there are some technical difficulties for standardizing the number and type of cells from bone marrow cultured *in vitro*. In our cases, the morphology of the peripheral blast cells was very similar to that of the blast cells in the bone marrow. If there is any leukemic cell sensitive to oxymetholone, this would possibly be a leukemic precursor whose activation should be very difficult to detect by short term cultures. Other methods as colony forming unit cells either *in vivo* or *in vitro* might be used.

SHAHIDI [16] has recently summarized the mechanisms of action of androgens on the erythropoietic cells The indirect action of androgens on erythropoiesis has been clearly established by many studies [1, 2, 9, 12], all showing consistently an increased level of endogenous erythropoietin This indirect action should not by itself produce any effect on leukemic cells On the other hand, several investigators have recently suggested a *direct* action of androgens on erythropoietic stem cells [8, 14] Certain 5β-H-steroids appreciably stimulate porphyrin and hemoglobin synthesis [11, 13] This effect is presumably achieved by stimulating δ-aminolevulinic synthetase, the rate-limiting enzyme for heme synthesis Evidently, this type of direct action could not affect leukemic cells

Some reports suggest that androgen metabolites trigger the colony-forming unit cells into the cell cycle [4, 15] Thus, by triggering the pluripotential stem cells from $G_0$ phase or prolonged $G_1$ phase into a $G_1$ interval responsive to erythropoietin, a greater number of erythropoietin-sensitive cells would be available to the increased level of erythropoietin This could be the only way by which androgen metabolites might affect directly acute leukemia cells or their precursor cells, and, at least in our study, we have not been able to demonstrate this direct effect on the peripheral acute leukemia blast cells

Recently, the development of hepatocellular 'hepatoma' or 'hepatocarcinoma' in patients with aplastic anemia treated with androgens has been reported [3, 10] Although there is no clear evidence of a definitive malignant transformation of normal hepatocytes by androgens, the clinical observation points to another possible deleterious effect of long-term androgen therapy On the contrary, a recent report [7] suggests that androgen might trigger cells into cycles that respond to differentiating factors (erythropoietin), thus keeping the patient in a controlled preleukemia stage

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# Glycopeptides of Erythrocyte Membranes in Some Hematological Disorders

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*Key Words* Dyserythropoietic anemia Erythrocyte membrane Glycopeptides PNH Polycythemia vera Sialic acid

*Abstract* Red cell membrane glycopeptides of subjects suffering from different hematological d sorders (PNH hemolytic anemias, dyserythropoietic anemias and polycythemia vera) have been characterized In most cases except in polycythemia vera a decrease of sialic acid and galactosamine was detected The role of these alterations is discussed with regard to the decrease of membrane glycopeptides during physiological aging of the red cell

Many authors have investigated the essential role of glycoproteins located at the external surface of the erythrocyte membrane [7, 10, 17–19] Sialic acid, a component of glycoproteins, was studied in particular and removal of this component from the membrane modifies some properties of the erythrocyte, like agglutination by viruses [11], antigenic characteristics [14], and life span [5, 6]

In our institute it has recently been shown that during the aging of erythrocytes in circulation, sialic acid and galactosamine are removed from membrane glycoproteins [1] On the basis of this evidence it was decided to investigate if the modifications which appear during physiological aging might also be present in some hematological disorders characterized by shortened red cell survival Erythrocyte membranes of patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) were also studied considering that normal erythrocytes after neuraminidase treatment are similar to PNH cells and in particular become susceptible to the hemolytic action of serum complement [20] A case of polycythemia vera was

also investigated on the basis of the hypothesis that in this disease a higher degree of young erythrocytes is present in the circulation [2]

## *Materials and Methods*

We studied glycopeptides extracted from erythrocyte membranes in 2 patients affected by congenital dyserythropoietic anemia type II (HEMPAS, cases 1, 2) and in 4 patients with sideroblastic anemia (cases 3–6). The red cell survival time was shortened in all these patients. Two cases of PNH were also studied (cases 7, 8). These patients had no severe hemolytic episode in the last 2 weeks. We also investigated erythrocyte membranes in 1 patient affected by polycythemia vera (case 9) and in two brothers, 43 and 41 years old, suffering from muscular dystrophy and severe hemolytic anemia from birth (cases 10, 11). The clinical features were similar in both cases: apart from pallor and mild splenomegaly no physical abnormalities were present. Laboratory investigations showed: (a) considerable normochromic normocytic anemia with a slightly elevated reticulocyte count; (b) no intrinsic or extrinsic red cell abnormalities could be demonstrated; (c) in particular, the enzymes of glycolysis, hexosemonophosphate shunt and other clinically significant enzymes were within the normal values; (d) no pathological hemoglobins were detectable, and (e) warm and cold antibodies were not present. The red cell survival was slightly shortened. All patients had no therapy or blood transfusion in the last month. The reticulocyte count was normal in cases 3, 4 and 9 and increased in the other patients (14‰ in case 1, 20‰ in 2, 11‰ in 5, 12‰ in 6, 15‰ in 7, 12‰ in 8, 9‰ in 10 and 10‰ in 11).

*Preparation of red cell ghosts.* A slight modification of the method of Dodge *et al.* [4] was used. 40–50 ml of blood were collected from the veins of patients using 3.8% sodium citrate as anticoagulant. Plasma and buffy coat (white cells) were removed after centrifugation at 4 °C at 1 000 *g* for 15 min and erythrocytes were washed three times with 10 vol of isoosmotic (310 mosm) sodium phosphate buffer, pH 7.4. Packed erythrocytes were then suspended in 4 vol of 20 mosm sodium phosphate buffer, pH 7.4 and maintained at 4 °C for 12 h. Ghosts were then sedimented by centrifugation at 20 000 *g* at 4 °C for 40 min and washed with 20 mosm sodium phosphate buffer, pH 7.4, until the supernatant was colorless, to remove as much hemoglobin as possible. A partial lipid extraction was carried out by suspending the sediment in 10 vol of ethanol-diethyl ether (3:1, v/v) kept stirring for 5 at 4 °C [12]. The treatment was repeated twice and the ghosts were then washed with 99% (v/v) ethanol and then ether.

*Extraction of glycopeptides from red cell ghosts.* Glycopeptides were extracted from membranes by papain digestion (1 U of papain/mg of dry ghosts) in 0.1 M sodium acetate buffer, pH 5.5, containing 5 mM EDTA and 5 mM cysteine at 65 °C for 45 h [13]. After heat inactivation of the enzyme and removal of the insoluble particles, the solution was dialyzed against bidistilled water and glycopeptides were precipitated in the presence of 0.6 M sodium acetate and 0.5 M acetic acid at 4 °C by the addition of 3 vol of 95% (v/v) ethanol [1]. No sialic acid was detectable in the supernatant.

*Table I* Sialic acid and hexosamine content in glycopeptides extracted from erythrocyte ghosts of 6 patients affected by dyserthropoietic anemias

| | Normal values | | Dyserythropoietic anemias | | | | | |
|---|---|---|---|---|---|---|---|---|
| | young ghosts | old ghosts | HEMPAS | | acquired | | | |
| | | | 1 | 2 | 3 | 4 | 5 | 6 |
| Sialic acid | 13.7±1.2 | 9.9±2.1 | traces | 3.0 | 7.5 | 4.1 | 8.3 | 13.7 |
| Galactosamine | 5.1±0.1 | 4.4±0.1 | traces | 2.0 | 2.5 | 2.4 | ND | 7.6 |
| Glucosamine | 13.2±1.9 | 14.0±2.4 | 13.2 | 10.9 | 11.5 | 21.7 | ND | 15.2 |

Normal values are means ± SD of 4–6 experiments carried out on pools of 2–3 donors each [1] Values are expressed as g/100 g dry weight

*Analytical methods* Hexosamines were determined with the Hitachi Perkin Elmer Liquid Chromatograph [9] Sialic acid was measured by the method of SVENNERHOLM [15] hexoses by the method of TREVELYAN and HARRISON [16] fucose by the method of DISCHE and SHETTLES [3] and total protein by the method of LOWRY *et al* [8]

## *Results*

*Dyserythropoietic anemia* Table I shows a significant decrease of sialic acid in three cases of acquired dyserythropoietic anemia, galactosamine concentration is also lower than in normal ghosts in cases 3 and 4, while glucosamine content shows considerable variations In a fourth patient (No 6) with acquired dyserythropoietic anemia the levels of sialic acid, glucosamine and galactosamine were among the normal values The determination of fucose, hexose and protein was carried out only on glycopeptides of case 3, owing to the poor blood availability of the three other patients, and did not show any variation from normality Protein and sugar composition was also normal in glycopeptides extracted from the ghosts of a patient firstly considered as affected by acquired dyserythropoietic anemia, but then resulting in a transient stage of erythroblastic hyperplasia during the recovery process of bone marrow aplasia In the first of the two cases of congenital dyserythropoietic anemia type II (No 1) sialic acid and galactosamine are present only in traces in ghost glycopeptides, and in the second case (No 2) the content of these two components is also extremely low Protein, hexose, and fucose content are within normal values

*Table II* Glycopeptides isolated from erythrocyte ghosts of patients suffering from PNH polycythemia vera and unknown hemolytic anemia

| | Normal values | | PNH | | Polycythemia vera | Hemolytic anemia and muscular dystrophy | |
|---|---|---|---|---|---|---|---|
| | young ghosts | old ghosts | 7 | 8 | 9 | 10 | 11 |
| Sialic acid | 13 7 ± 1.2 | 9.9 ± 2.1 | 4 0 | traces | 12.3 | traces | traces |
| Galactosamine | 5 1 ± 0 1 | 4 4 ± 0 1 | 0 8 | traces | 5 6 | 3 0 | 1 0 |
| Glucosamine | 13 2 ± 1 9 | 14 0 ± 2.4 | 17 0 | 9.5 | 11 4 | 22.7 | 23 4 |
| Fucose | 4 1 ± 0 1 | 3 0 ± 0 1 | ND | ND | 2.4 | 5.2 | 4.2 |
| Hexose | 23 2 ± 0 1 | 23.2 ± 2.2 | ND | ND | 20 4 | 24 0 | 22.0 |
| Protein | 24 6 ± 2.2 | 25.5 ± 2.0 | ND | ND | 30 4 | 20 0 | 14 0 |

Values are expressed as g/100 g dry weight.

*Paroxysmal nocturnal hemoglobinuria.* Glycopeptides extracted from the PNH erythrocyte ghosts show an extremely low content of sialic acid and galactosamine (table II) Particularly in case No 8, these two components are not detectable

*Hemolytic anemia with muscular dystrophy* As reported above two brothers were affected by muscular dystrophy associated with a serious form of hemolytic disorder which could not be framed in any of the known hemolytic diseases. Table II shows that in both cases sialic acid is not detectable in ghost glycopeptides The galactosamine content is also very low, while glucosamine in concentration is higher than normal Protein fucose and hexose were within normal values

*Polycythemia vera* In the only case of polycythemia very examined (table II) no variation with respect to the normal values was detected in ghost glycopeptides except a significantly higher protein content.

## *Discussion*

Mechanisms by which erythrocytes in normal and pathological conditions are removed from the circulating blood are only partially known Membrane structure certainly plays a fundamental role in the determination of red cell life span in particular it was recently pointed out that sial

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# Stimulation of Phagocytosis by Human Lysozyme

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*Key Words.* Lysozyme · Neutrophil leukocytes · Phagocytosis

*Abstract.* Human lysozyme in physiologic concentrations (10-400 μg/ml) significantly ($p<0.001$) stimulates the phagocytosis of yeast cells by human polymorphonuclear leukocytes. This stimulating effect was observed even in the absence of serum factors. Hen egg white lysozyme or protamine sulfate had no effect on phagocytosis. The stimulating effect of human lysozyme was not the result of opsonization but was apparently related to the effects on constituent membranes of the phagocytic cells.

The presence of lysozyme (LZM) in phagocytic cells, particularly polymorphonuclear neutrophils (PMN), monocytes and macrophages, has been well documented [4, 5]. LZM has generally been considered to participate in both intracellular and extracellular bactericidal processes, primarily in the later stages of the phagocytic process. Recent observations on the effects of LZM on mammalian cells and their cytoplasmic membranes [11] suggest the possibility that LZM might also participate in the earlier stages of phagocytosis in which major membrane activities are involved [13].

To gain further insight into this possible role of LZM, we have studied the effect of homologous human LZM on the *in vitro* phagocytosis of yeast cells by human PMN.

## *Material and Methods*

*Phagocytosis.* Buffy coat leukocytes were prepared by sedimentation from heparinized peripheral venous blood of healthy laboratory personnel. PMN were suspend

ed in autologous plasma (heparin) or in various media to a concentration of 5 000 cells/µl Phagocytic activity was determined by the method of BRANDT [2] using heat killed Baker s yeast cells cells in suspensions with a PMN to yeast cell ratio of 1 10 *After incubation for 30 min at 37 °C, the suspension was centrifuged and the* resulting pellet was smeared on glass slides and stained with May Grunwald Giemsa 100 PMN were examined, and the mean number of yeast cells ingested per PMN was calculated and expressed as the 'phagocytic index' (PI)

*Lysozyme* Human LZM was isolated from the urine of patients with monocytic or myelomonocytic leukemia by the bentonite adsorption method [12] followed by ion exchange chromatography on CM 32 resin (Whatman) with a linear elution gradient of 0 05–0 5 M $NaHCO_3$, pH 8 0 [3] Hen egg white LZM (crystallized dialyzed and lyophilized three times) and protamine sulfate (from herring, grade III) were purchased from Sigma Chemical Co

Removal of LZM from serum was done with bentonite adsorption The bentonite was first washed with large quantities of Dulbecco s phosphate buffered saline, pH 7 2, and then added to serum at a concentration of 10 mg/ml The suspension was thoroughly mixed and incubated at room temperature for 30 min The LZM concentration of the serum supernatant after centrifugation, as measured by the lysoplate method [12] was zero

*Opsonization* To test the opsonizing effect of human LZM, the yeast cells were preincubated with LZM, 400 µg/ml, for 30 min at room temperature, washed with PBS and tested for susceptibility to phagocytosis

*Gel electrophoresis* Polyacrylamide gel electrophoresis in 8% polyacrylamide containing 0 1% SDS was performed according to LAEMMLI [9]

## *Results*

The purity of human LZM was verified by polyacrylamide gel electrophoresis which demonstrated a single band (fig 1)

The PI of PMN obtained from 19 healthy persons was 4 6±0 3 (fig 2) When human LZM in concentrations of 10–400 µg/ml was added to the PMN suspended, as above, in *heparinized autologous plasma, there* was a significant increase in the ingestion of yeast cells The addition of 20 µg/ml of human LZM, for example, raised the PI to 5 4±0 4 ($p<0\ 001$) In contrast to the effect of human LZM, neither protamine sulfate nor hen egg white LZM stimulated phagocytosis No morphological changes in the PMN were visible by light microscopy, and no effect on the viability of the PMN in the presence of up to 400 µg/ml of LZM could be demonstrated by trypan blue dye exclusion

To determine the *PI of PMN incubated in LZM-free medium* (except for the endogenous LZM of PMN), the cells were washed three times with Medium 199 and suspended in autologous normal serum and in au-

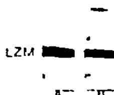



*Fig 1.* Polyacrylamide gel electrophoresis of 25 μg of human LZM (A) isolated by bentonite adsorption and chromatography on carboxymethyl cellulose (CM 32 resin) and hen egg white LZM (B) in 8% polyacrylamide slab gels containing 0.1% SDS according to LAEMMLI [8].

tologous serum from which LZM had been removed by bentonite adsorption (table I). The PI of PMN suspended in normal autologous serum was 5.1 ± 0.3. When the serum had been adsorbed with bentonite, the PI decreased to 4.5 ± 0.3 ($p<0.001$). The PI of PMN suspended in serum free or plasma free medium was reduced to 0.5 ± 0.2, but even in the absence of serum or plasma, human LZM stimulated phagocytosis to a PI of 0.9 ± 0.2 ($p<0.001$). A comparable decrease in the phagocytic activity of PMN was observed when the cells were suspended in heat inactivated serum. Even under these conditions, LZM apparently stimulated the phagocytic activity of PMN, although not to a statistically significant level.

When the PMN from 9 persons were exposed to untreated or LZM treated (opsonized) yeast cells, the PI was 4.7 ± 0.4 and 4.8 ± 0.3, respectively (not statistically significant).

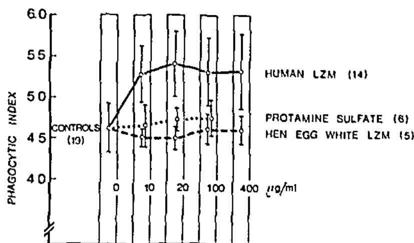



*Fig 2* PI (mean ± SD) of human PMN in the presence of 10–400 μg/ml of human LZM, hen egg white LZM and protamine sulfate In parentheses are the numbers of individuals tested for each agent

*Table I* Effect of LZM on the phagocytosis of yeast cells by human PMN

| Medium[1] | Phagocytic index mean±SD | Number of individuals tested | p value[2] |
|---|---|---|---|
| Autologous serum | 5 1±0 3 | 16 | <0 001 |
| Bentonite-adsorbed autologous serum | 4 5±0 3 | 13 | |
| Medium 199 | 0 5±0 2 | 10 | <0 001 |
| Medium 199 + human LZM, 100 μg/ml | 0 9±0 2 | 10 | |
| Heat inactivated autologous serum, +56°C for 15 min | 0 5±0 3 | 3 | not significant |
| Heat inactivated autologous serum + human LZM, 100 μg/ml | 0 6±0 3 | 3 | |

[1] Cells were washed three times in Medium 199 and thereafter suspended in the various media

[2] Student's t test

## *Discussion*

The initial steps in the phagocytic process involve the attachment of the material to be ingested to the external surface of the cell, the formation of pseudopodia and the phagocytic vesicle [13] Whether a microor-

ganism is engulfed depends on the interaction of several factors including opsonins (antibodies) complement and other heat labile factors and the cell membrane of the phagocyte [14] Previous studies [7] have suggested that LZM might act in certain circumstances as an opsonin in the phagocytosis of bacteria independently of the LZM sensitivity of the bacteria LYCKE *et al* [10] and SENECA and PEER [15] have also described an enhancing effect of high concentrations (1 000 μg/ml) of hen egg white LZM on the phagocytosis of *Toxoplasma gondii* by HeLa cells and of *Proteus vulgaris* and *Streptococcus haemolyticus* by human PMN With respect to the present studies it is particularly noteworthy that the stimulation of phagocytosis was demonstrated only with the homologous human LZM and that the concentrations used are probably within the range achieved in inflammatory exudates *in vivo* [16]

Time sequence studies on the degranulation of PMN that accompanies phagocytosis have shown that the initial events are related to changes in those specific granules that contain most of the LZM in the PMN [6 17] LZM is released into the extracellular medium early during phagocytosis without any accompanying appreciable rise in enzyme activity in the cytoplasm or any indication of cellular damage We might therefore, postulate that this release of LZM affects a local milieu of autostimulation of phagocytosis in the very early stages of the phagocytic process

The present studies indicate that LZM does not act as a true opsonin since its action is on the phagocyte and not the target organism Although LZM stimulated phagocytosis in serum free media the significance of serum factors in the engulfment process was apparent Whether the decrease in PI of PMN suspended in heparinized plasma as compared with cells suspended in serum was related to the 30–40% inhibition of LZM activity caused by heparin needs further investigation [5 and unpublished observations]

The cationic LZM molecule in addition to causing structural changes in certain cell membranes [11] could possibly also function as glue' in the process of particle attachment Whereas adhesion *per se* of particles to the PMN surface does not produce maximal ingestion such adhesion probably promotes ingestion both in the absence or presence of serum factors When serum IgG participates it appears that besides promoting or stimulating particle attachment it also triggers the ingestion step [14]

Thus far no specific substrate for LZM in mammalian cells has been identified The stimulating effect of species-specific LZM on the phagocytic activity of PMN could not be achieved with LZM isolated from hen

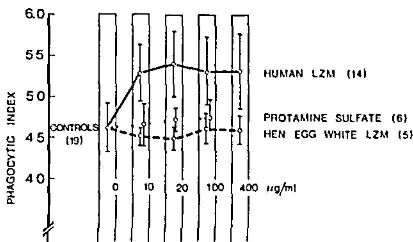



*Fig 2* PI (mean ± SD) of human PMN in the presence of 10–400 μg/ml of human LZM hen egg white LZM and protamine sulfate In parentheses are the numbers of individuals tested for each agent

*Table I* Effect of LZM on the phagocytosis of yeast cells by human PMN

| Medium[1] | Phagocytic index mean±SD | Number of individuals tested | p value[2] |
|---|---|---|---|
| Autologous serum | 5 1±0 3 | 16 | <0 001 |
| Bentonite adsorbed autologous serum | 4 5±0 3 | 13 | |
| Medium 199 | 0 5±0 2 | 10 | <0 001 |
| Medium 199+human LZM 100 μg/ml | 0 9±0 2 | 10 | |
| Heat inactivated autologous serum +56°C for 15 min | 0 5±0 3 | 3 | not significant |
| Heat inactivated autologous serum + human LZM 100 μg/ml | 0 6±0 3 | 3 | |

[1] Cells were washed three times in Medium 199 and thereafter suspended in the various media

[2] Student s t test

## *Discussion*

The initial steps in the phagocytic process involve the attachment of the material to be ingested to the external surface of the cell, the formation of pseudopodia and the phagocytic vesicle [13] Whether a microor-

ganism is engulfed depends on the interaction of several factors including opsonins (antibodies), complement and other heat labile factors and the cell membrane of the phagocyte [14]. Previous studies [7] have suggested that LZM might act in certain circumstances as an opsonin in the phagocytosis of bacteria independently of the LZM sensitivity of the bacteria. LYCKE *et al.* [10] and SENECA and PEER [15] have also described an enhancing effect of high concentrations (1 000 $\mu$g/ml) of hen egg white LZM on the phagocytosis of *Toxoplasma gondii* by HeLa cells, and of *Proteus vulgaris* and *Streptococcus haemolyticus* by human PMN. With respect to the present studies it is particularly noteworthy that the stimulation of phagocytosis was demonstrated only with the homologous human LZM and that the concentrations used are probably within the range achieved in inflammatory exudates *in vivo* [16].

Time sequence studies on the degranulation of PMN that accompanies phagocytosis have shown that the initial events are related to changes in those specific granules that contain most of the LZM in the PMN [6, 17]. LZM is released into the extracellular medium early during phagocytosis without any accompanying appreciable rise in enzyme activity in the cytoplasm or any indication of cellular damage. We might, therefore, postulate that this release of LZM affects a local milieu of 'autostimulation' of phagocytosis in the very early stages of the phagocytic process.

The present studies indicate that LZM does not act as a true opsonin since its action is on the phagocyte and not the target organism. Although LZM stimulated phagocytosis in serum free media, the significance of serum factors in the engulfment process was apparent. Whether the decrease in PI of PMN suspended in heparinized plasma, as compared with cells suspended in serum, was related to the 30–40% inhibition of LZM activity caused by heparin needs further investigation [8 and unpublished observations].

The cationic LZM molecule, in addition to causing structural changes in certain cell membranes [11] could possibly also function as 'glue' in the process of particle attachment. Whereas adhesion *per se* of particles to the PMN surface does not produce maximal ingestion, such adhesion probably promotes ingestion both in the absence or presence of serum factors. When serum IgG participates, it appears that, besides promoting or stimulating particle attachment, it also triggers the ingestion step [14].

Thus far, no specific substrate for LZM in mammalian cells has been identified. The stimulating effect of species-specific LZM on the phagocytic activity of PMN could not be achieved with LZM isolated from hen

egg white, and presently available evidence indicates that hen and human LZM have the same substrate specificity and cationic properties There are, however, significant differences in the primary and tertiary structures of these two enzyme [1], and these may be involved in the stimulating effect of human LZM on phagocytosis

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# Salicylamide-Glucuronide Formation in Children with Favism and in Their Parents

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*Key Words* Favism · G 6-PD deficiency · Liver function in favism · Salicylamide-glucuronide

*Abstract* Salicylamide glucuronide formation has been determined in 27 children who underwent a favism crisis, 25 parents, and in 25 normal children who served as controls. A highly significant mean lower glucuronide formation was observed in the favism group in respect to the controls. The difference between fabic children and their parents was significant, and between parents and controls there was no significant difference.

A deficiency in the G-6-PD activity of mature red cells leads to hemolytic crises after ingestion of fava beans, administration of some drugs or, more rarely, in the course of viral hepatitis. An increased red cell turnover [1] or severe hemolysis [2] have been reported in isolated cases of hyperbilirubinemia in newborns with such a deficiency, but research we performed on a large casuistry of mature newborns, demonstrated that this form of severe neonatal jaundice is 'paradoxically' not hemolytic in origin. We postulated a possible liver involvement associated with G-6-PD deficiency which deteriorates the postnatal transiently impaired liver ability to metabolize bilirubin [3]. *The idea that G-6-PD deficiency could be considered* as a cause of metabolic impairment other than in erythrocytes received experimental support, as it was demonstrated that salicylamide-glucuronide formation is significantly decreased in enzyme-deficient hyperbilirubinemic newborns [4] and in children who experienced a favism crisis [5].

In the present paper the problem of salicylamide-glucuronide formation has been reexamined in a group of children with favism and in their parents.

## Materials and Methods

Salicylamide glucuronide formation was determined in 27 children (13 males and 14 females, 7 of whom were heterozygotes) whose age ranged between 2 and 9 years, admitted to the clinic after a hemolytic crisis due to fava bean ingestion. Hemoglobinuria occurred in all cases and anemia was more or less pronounced (Hb range 5–8.2 g%). The latter was corrected within 24–72 h from admittance by two or more blood transfusions. Salicylamide was administered 2 or 3 days after the last transfusion and the Hb level was in no case less than 10.5 g%. The test was also carried out on some volunteers among the parents of the affected children: 8 affected fathers of females and 17 mothers (10 of whom were homo- and 7 heterozygotes). 25 healthy children without hematological traits aged 6–12 years served as controls. Diagnosis of G-6-PD deficiency was made in all cases, using the cytochemical test of SANSONE *et al.* [6], on venous blood which was taken from the affected children immediately before blood transfusion. The salicylamide (Urtosal Heumann) test was performed by giving a single 20 mg/kg body weight oral dose of salicylamide diluted in water in the morning after breakfast, and urine was collected during the next 24 h. Total salicylamide and salicylamide-glucuronide were determined by the method of LEVY and MATSUZAWA [7].

## Results

The mean salicylamide-glucuronide formation was 31.25 ± 20.49% in the fabic children, 49.59 ± 19.94% in their parents and 56.18 ± 11.30% in the control subjects. The difference between fabic and normal children was highly significant ($p < 0.001$). No significance was found between parents and normal children ($p > 0.1$) and little significance between fabic children and their parents ($p < 0.01$).

## Discussion

The data show that in children with favism the mean glucuronide formation is significantly lower than in normal subjects, thus confirming the results of CASSIMOS *et al.* [5] who found that glucuronide formation was less than 23% (arbitrarily established as the borderline between normal and affected persons) in 12 of 18 cases. In our favism group in 16 of 27 (59%) the glucuronide formation was lower than 33% (the minimum value found in the normal subjects). The mean values of the parents were not statistically different from the controls, however, in 7 of 25 (28%) of the former the values were lower than 33% and 5 of these had experienced a favism crisis at some time or other. It is noteworthy that no difference was observed be-

*Table I* Salicylamide-glucoronide excretion in urine (% of total) in children who experienced a favism crisis, in a group of their parents, and in controls

| Children with favism | | | Parents | | | Controls | | |
|---|---|---|---|---|---|---|---|---|
| No | sex | urine excretion | No | sex | urine excretion | No | sex | urine excretion |
| 1 | ma | 22 2 | 1 | fe ho | 54 8 | 1 | ma | 41 6 |
| 2 | fe he | 17 0 | 2 | fe he | 45 2 | 2 | ma | 47 0 |
| 3 | fe ho | 63 2 | 3 | fe he | 70 7 | 3 | ma | 61 5 |
| 4 | fe he | 18 4 | 4 | ma | 87 6 | 4 | fe | 55 5 |
| 5 | fe-ho | 29 2 | 5 | fe-he | 71 5 | 5 | fe | 45 2 |
| 6 | ma | 41 1 | 6 | fe he | 20 8 | 6 | fe | 61 4 |
| 7 | fe-he | 10 8 | 7 | fe ho | 56 7 | 7 | fe | 51 5 |
| 8 | ma | 54 8 | 8 | ma | 33 3 | 8 | ma | 68 7 |
| 9 | fe he | 75 4 | 9 | ma | 33 3 | 9 | fe | 80 2 |
| 10 | fe-ho | 6 6 | 10 | fe ho | 25 8 | 10 | ma | 49 0 |
| 11 | fe-he | 6 6 | 11 | fe ho | 38 8 | 11 | ma | 66 2 |
| 12 | ma | 39 0 | 12 | ma | 55 4 | 12 | ma | 33 4 |
| 13 | ma | 42.0 | 13 | fe ho | 79 2 | 13 | ma | 61 6 |
| 14 | fe ho | 5 9 | 14 | ma | 32.1 | 14 | ma | 60 0 |
| 15 | ma | 32 7 | 15 | ma | 76 3 | 15 | fe | 70 3 |
| 16 | fe ho | 8 8 | 16 | fe-he | 46 4 | 16 | fe | 67 8 |
| 17 | fe he | 44 5 | 17 | ma | 48 3 | 17 | ma | 53 3 |
| 18 | ma | 32 0 | 18 | fe ho | 62 5 | 18 | ma | 60 4 |
| 19 | fe ho | 57 3 | 19 | fe-ho | 28 4 | 19 | fe | 41 8 |
| 20 | ma | 68 8 | 20 | ma | 41 6 | 20 | fe | 63 1 |
| 21 | ma | 34 2 | 21 | fe ho | 66 4 | 21 | ma | 53 1 |
| 22 | fe ho | 19 0 | 22 | fe ho | 8 2 | 22 | ma | 57 1 |
| 23 | fe he | 50 0 | 23 | fe ho | 64 4 | 23 | ma | 36 8 |
| 24 | ma | 8 6 | 24 | fe he | 53 3 | 24 | ma | 66 0 |
| 25 | ma | 28 5 | 25 | fe he | 36 7 | 25 | ma | 51 1 |
| 26 | ma | 16 2 | | | | | | |
| 27 | ma | 12 2 | | | | | | |
| Mean | | 31 35 | | | 49 59 | | | 56 18 |
| SD | | 20 49 | | | 19 94 | | | 11 30 |

ma = Male, fe ho = female homozygous fe he = female heterozygous

tween the homo and heterozygotes with respect to glucuronide activity Furthermore, it should be stressed that low glucuronide activity was parallel-ed between affected children and their parents only in 4 cases 1 male, 1 heterozygous and 2 homozygous females No such parallelism was found in the remainder

An overall consideration of these data points to a probable link which has not yet been clarified between G-6-PD deficiency and liver ability in glucuronizing salicylamide. What relationship, if any, this defect may bear to favism crises is only a matter of speculation, whereas it seems possible that there is a connection between this impaired liver ability as demonstrated in the neonatal age too [4, 8] and hyperbilirubinemia in mature newborns.

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# Blood Group Phenotypes and Hemoglobin S

## An Anthropologic Study in Two Israeli Arab Communities

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*Key Words* Anthropology Blood groups Hemoglobin S Israeli Arabs

*Abstract* Blood group phenotypes of anthropologic significance are described for inhabitants of two Israeli Arab communities with foci of hemoglobin S (HbS) The presence of Fy(a–b–) $Rh_0$ $hr^{V*}$, and Js(a+) among the Hulah Valley Bedouin and of Fy(a–b–), $Rh_0$ and $hr^{V*}$ in Acre are indicative of genetic admixture of African origin These non African foci of HbS are thereby distinguished from previously described HbS foci in India Greece, and Turkey where low or absent $Rh_0$ phenotypes imply secondary dispersions of the HbS gene from the Arabian peninsula

The present distribution of sickle cell hemoglobin (HbS) in non-black populations of Europe, Asia, and the Americas is attributable either to *multiple mutations at the HbS locus, or more plausibly, to a single mutation* whose dispersion reflects historical population migrations [7] Genetic and historic evidence favor an equatorial African origin for the HbS mutation which diffused east to Arabia, and subsequently to Europe and Asia [8] An alternative hypothesis, which emphasized the relatively low frequency of the African blood group phenotype $Rh_0$ (cDe) in HbS foci of Greece, Turkey, and India, proposes that the HbS mutant arose in Saudi Arabia, spreading west to Africa, north to Europe, and east to India [12] In either instance, HbS among non-black populations of Europe and Asia appears to represent secondary dispersions from Arabia, and the persistance of HbS in high concentrations in these isolated foci probably

reflects local selective factors including endemic malaria [16] and G6PD deficiency [14].

The following report describes the blood group anthropology of two communities of Israeli Arabs with HbS. In contrast to previously described studies of HbS among European and Asian populations, the present report documents a remarkably high correlation of HbS with specific blood group phenotypes, indicating the genetic individuality of non-African foci of HbS.

## Materials and Methods

*Hulah Valley Bedouin.* In the first of two population surveys, specimens were obtained from 132 Bedouin representing 17 Arab families who reside in the Hulah Valley north of the Sea of Galilee. The region was endemic for *P. falciparum* and *P. vivax* until the mid 1930s. The origin of the Hulah Valley Bedouin is obscure but it is most probable that their ancestors migrated several hundred years ago from the area that is presently identified as northern Iraq. In physical appearance, the Hulah Valley Bedouin are not overtly Negroid and are indistinguishable from Arabs of neighboring villages. Sickle cell disease in these Bedouin was first described in 1955 [19].

*Acre.* The second population survey consisted of specimens from 32 urban Arabs, representing 8 families from the coastal city of Acre. Sickle cell disease in this community was first detected in 1973 and the clinical details as well as those for the Hulah Valley Bedouin have been described elsewhere [22].

The propositi, two children with sickle cell disease (HbSS), were not obviously Negroid in physical appearance. However, in the course of the field study, and with the knowledge that HbS was present in the community, the authors did note Negroid features among other inhabitants of the community.

*Blood group and hemoglobin typing.* In addition to routine ABO and Rh blood group phenotyping, fresh specimens were tested for the following antigens: M, N, S, s, $Fy^a$, $Fy^b$, $Js^a$, and $hr^V$ (V). The red cell phenotypes Fy(a−b−), $Rh_0$ (cDe), Js(a+), and $hr^V$+ are individually almost diagnostic of African origin [4, 21]. Combinations of these antigens without evidence of African ancestry have not been described. Fy(a−b−), probably the most reliable African blood group phenotype, was not present among 9,167 Caucasians [13], but has been detected in 80-90% of West Africans [21].

$Rh_0$, occurring in 2-3% of Caucasians, is found in 50-90% of African tribesmen [20]. The antigens $hr^V$ (V) and Js(a+) occur in less than 1% of Caucasians, but in 47% [5] and 29% [9], respectively, of West Africans. There are no published data for the frequencies of phenotypes Fy(a−b−), $Rh_0$ and Js(a+) for the indigenous Arab populations of Israel. However, the presence of phenotypes $Rh_0$, Fy(a−b−) and $hr^V$ in South Sinai Bedouin [3], $Rh_0$ and $hr^V$+ in Saudi Arabia [15], and $Rh_0$ in Sephardi [17], North African [10], and Arabian Jews [2] are all considered to reflect African admixture.

*Table I* Relationship of specific blood group phenotypes and hemoglobin types in two communities with foci of *HbS*

| Blood group Phenotype | Fy (a–b–) | $Rh_o$ | Js (a+) | $hr^{V}$+ | Fy (a–b–) $Rh_o$ | Fy (a–b–) Js (a+) | Fy (a–b–) $hr^{V}$+ | $Rh_o$ $hr^{V}$+ | Fy (a–b–) $Rh_o$ $hr^{V}$+ | Fy (a–b–) $Rh_o$ Js (a+) | None | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Hulah Valley* | | | | | | | | | | | | |
| HbSS | 5 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 11 |
| HbAS | 22 | 0 | 2 | 0 | 2 | 5 | 1 | 1 | 0 | 1 | 6 | 40 |
| HbAA | 36 | 0 | 0 | 0 | 4 | 4 | 1 | 0 | 0 | 0 | 36 | 81 |
| *Acre* | | | | | | | | | | | | |
| HbSS | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 5 |
| HbAS | 9 | 1 | 0 | 0 | 2 | 0 | 1 | 0 | 3 | 0 | 1 | 17 |
| HbAA | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 6 |
| HbCC | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| HbAC | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |

*Table II* Distribution and frequencies of selected blood group phenotypes

| Phenotype | West African[1] blacks, % | Caucasians[1] % | Hulah Valley | | Acre | |
|---|---|---|---|---|---|---|
| | | | % | number | % | number |
| Fy (a- b-) | 90 | 0 | 62 | 82 | 88 | 28 |
| $Rh_o$ | 60 | 3 | 7 | 9 | 38 | 12 |
| Js (a+) | 16 | 0 | 11 | 14 | 0 | 0 |
| $hr^{V}$+ | 40 | 0.5 | 2 | 3 | 25 | 8 |

[1] Data summarized from previous surveys [4, 21].

At the time of testing, the only anti $hr^{V}$+ reagent available was specific for type O red cells and, therefore, the $hr^{V}$+ results reflect testing of only type O specimens.

Hemoglobin types were determined by starch gel electrophoresis and in homozygotes, by the appearance of sickle cells in fresh peripheral blood smears. Hemoglobin typings were confirmed in the laboratory of Dr. T. H. HUISMAN, Atlanta, Ga.

## *Results*

*Hulah Valley.* The hemoglobin types of 132 Hulah Valley Bedouin (representing 17 families) were HbSS (11), HbAS (40) and HbAA (81) (table I). African blood group markers were prevalent. 66% of the sampling had at least one of the specific blood group phenotypes and 16% had two or more. 62% of the sampling were Fy(a-b-), 7% were $Rh_o$, 11% were Js(a+), and 2% were $hr^{V}$+ (table II).

*Acre.* The hemoglobin types of 32 Acre Arabs (representing 8 families) were HbSS (5), HbAS (17), HbAA (6), HbCC (1), and HbAC (3) (table I). In this sampling 94% had at least one of the specific blood group phenotypes and 38% had two or more. Although the phenotype Js(a+) was not present, the other specific blood group phenotypes were more prevalent than in the Hulah Valley (table II). 88% were Fy(a-b-), 38% were $Rh_o$, and 25% $hr^{V}$+.

## *Discussion*

The present known distribution of HbS in non-blacks includes inhabitants of Greece, Sardinia, Italy, Turkey, Libya, Tunisia, Iran, Saudi Arabia, India and Southeast Asia [1]. HbS has not been found in Israeli

Jews [24], but has been described previously in Israeli Arabs [19]. HbS has been reported in the United States in Caucasians with no apparent black ancestry [11], where the incidence may be as high as 0.08% [18].

The present report illustrates the role of blood group anthropology in the study of HbS in populations and in individuals without physical or known historical evidence of African ancestry. The presence of phenotypes Fy(a–b–), $Rh_0$, Js(a+) and $hr^{V+}$ in the Hulah Valley Bedouin and Fy(a–b–), $Rh_0$ and Js(a+) in Acre, documents direct African admixture. The fortuitous finding of HbCC and AC in Acre is additional evidence of genetic admixture from West Africa, since HbC is highly localized to West Africa [6] and is not found elsewhere without evidence of African ancestry. The four individuals with HbC in the present study are relatives of a large family of Bedouin with a known high incidence of HbC [23].

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Jews [24], but has been described previously in Israeli Arabs [19]. HbS has been reported in the United States in Caucasians with no apparent black ancestry [11], where the incidence may be as high as 0.08% [18].

The present report illustrates the role of blood group anthropology in the study of HbS in populations and in individuals without physical or known historical evidence of African ancestry. The presence of phenotypes Fy(a–b–), $Rh_o$, Js(a+) and $hr^{V+}$ in the Hulah Valley Bedouin and Fy(a–b–), $Rh_o$ and Js(a+) in Acre, documents direct African admixture. The fortuitous finding of HbCC and AC in Acre is additional evidence of genetic admixture from West Africa, since HbC is highly localized to West Africa [6] and is not found elsewhere without evidence of African ancestry. The four individuals with HbC in the present study are relatives of a large family of Bedouin with a known high incidence of HbC [23].

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# Effect of Hyperoxia and Androgen on Red Cell 2,3-Diphosphoglycerate and Oxygen Affinity[1]

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*Key Words* Androgens 2 3 Diphosphoglycerate Erythropoietin Hyperoxia Oxygen dissociation Oxyhemoglobin affinity

*Abstract* *Increased plasma erythropoietin (ESF) activity, a rise in RBC 2,3-DPG,* and a 'right shift' of the oxygen hemoglobin equilibrium curve following androgen administration to mice in ambient or hyperoxic conditions was demonstrated If androgens had a direct effect on the RBC metabolism increased 2,3-DPG would *result in a facilitated release of oxygen to the tissue This would have been* accompanied by a decrease rather than an increase in the level of ESF Hyperoxia has abolished detectable rising levels of plasma ESF and RBC 2 3 DPG following androgen administration These levels were close to those seen in the ambient nontreated mice

*The erythropoietic capacity of androgens has long been recognized* [13, 16, 35] It has been clearly demonstrated [1, 13] that androgens are capable of stimulating erythropoietin (ESF) production Recent studies reported a rise in RBC 2,3 diphosphoglycerate (2,3-DPG) and a 'right shift' of the oxygen hemoglobin equilibrium curve in hypoxic exposure [41] or following androgen administration in monkeys [18] and in patients with chronic renal failure maintained on bi-weekly dialyses [42] Two mechanisms may be proposed to account for this rise in RBC 2,3-DPG following androgen administration a direct effect of the androgen on the metabolism of the RBC, and/or relative tissue hypoxia. Hyperoxia has been reported to suppress RBC 2,3-DPG levels [25] The experi-

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ments reported here were designed to determine the effect of hyperoxia on the changes noted in plasma ESF levels, RBC 2,3-DPG, and the oxygen hemoglobin equilibrium curve following androgen administration.

## Materials and Methods

*Animals* Swiss Webster white virgin female mice (West Jersey Biological Co., Wenonah N.J.) randomized in groups of ten, were used and supplied with Purina Rodent Chow and water, *ad libitum* The mice received 1-2 mg of iron as iron-dextran solution (Imferon®, Lakeside Laboratories, Milwaukee, Wisc.) intraperitoneally to insure adequate iron stores.

*Hyperoxic chamber* Endogenous ESF, as measured by radioiron incorporation into peripheral RBC and reticulocyte counts [3] was suppressed by maintaining mice in a modified atmosphere chamber (Piersol Pine Manufacturing Co., Oaks, Pa.) at 60% oxygen concentration and normal atmospheric pressure [27] Temperature was maintained as close as possible to 22 °C. The exposure to hyperoxia was interrupted daily for 10-15 min cage cleaning and injections.

Pooled blood from a group of 7 mice was obtained following 6 days exposure to hyperoxia and 48 h after the second 19-nortestosterone decanoate (19-ND) injection and RBC 2,3-DPG and the partial pressure at which 50% of the hemoglobin is oxygenated ($P_{50}$) were measured according to the method of Delivoria Papadopoulos *et al* [7] Nortestosterone decanoate was kindly supplied by Dr Henry Strade (Organon West Orange, N.J.) in a concentration of 25 mg/ml dissolved in propylene glycol Injections were given on the 4th and 5th hyperoxic day

*Determination of plasma erythropoietic activity in the posthypoxic plethora.* Erythropoietic activity in plasma of mice at ambient pressure was determined 1 h after the administration of a single injection of 2.5 mg 19 ND and compared to the activity 3 days later Plasma for ESF determination was also obtained from hyperoxic mice 1 2, 3, and 4 days after two daily administrations each of 2.5 mg 19 ND dissolved in 0.1 ml of propylene glycol Plasma ESF levels were measured in the posthypoxic polycythemic mouse system by injecting plasma samples of 0.5 or 1.0 ml each to mice on the 3rd and 5th hypoxic days, and 72 h radioiron incorporation was begun on the 6th posthypoxic day as previously described [19 23]

## Results

The results of $^{59}$Fe incorporation into RBC of posthypoxic polycythemic mice (table I) clearly indicate the lack of ESF activity of plasma obtained 1, 2, 3, and 4 days after 19 ND administration to mice maintained in hyperoxia. Radioiron incorporation into RBC of posthypoxic polycythemic mice following administration of plasma obtained from mice maintained in normal conditions and injected with 19 ND 1 h or 3

*Table I* Percent $^{59}Fe$ incorporation in posthypoxic polycythemic mice following administration of plasma obtained from mice maintained in hyperoxia and injected with 19-ND

| Plasma obtained, days after 2nd 19-ND injection | Amount of plasma injected each time, ml | $^{59}Fe$ incorporation, %[1] |
|---|---|---|
| 1 | 0 5 | 2 5±0 7 |
| | 1 0 | 2.6±0 8 |
| 2 | 0 5 | 1.9±0 6 |
| | 1 0 | 3 0±1 0 |
| | 0 5 | 1.9±0 4 |
| 3 | 1 0 | 1 7±0 7 |
| 4 | 0 5 | 1 7±0 7 |
| | 1 0 | 2 2±0 5 |
| Control[2] | | 2 0±1 2 |
| 0 25 U ESF[3] | | 4 1±0 3 |

[1] Results expressed as mean±SEM of a group of at least eight animals
[2] Injection of diluent only
[3] Injected each time as for plasma administration

*Table II* RBC 2,3-DPG and $P_{50}$ values in ambient and hyperoxic mice before and following 19-ND administration

| | | 2,3-DPG, nM/ml RBC | $P_{50}$ mm Hg, pH 7 4 | DPG[1] | $P_{50}$[1] | DPG[2] | $P_{50}$[2] |
|---|---|---|---|---|---|---|---|
| Before | ambient | 8,900±157 | 40 5±0 25 | <0 05 | | | |
| 19-ND | hyperoxic | 6,100±203 | 39 0±0 25 | | <0 1 | | |
| Following | ambient | 10,250±215 | 42.5±0 25 | <0 05 | | <0 05 | |
| 19-ND | hyperoxic | 9,050±250 | 41 0±0 25 | | <0 1 | | <0 05 |

Each point represents mean of at least four measurements ±SEM, each measurement performed on pooled blood

[1] Statistical significance of change in RBC, 2,3-DPG levels or $P_{50}$ position following hyperoxic exposure

[2] Statistical significance of change in RBC 2,3-DPG levels or $P_{50}$ position following administration of 19 ND to mice in hyperoxic exposure

days before collection of plasma were previously reported [20] The results of the RBC 2,3 DPG and $P_{50}$ (table II), clearly indicate a decrease in RBC 2,3 DPG as previously reported [25] along with a 'shift to the left' of the oxygen hemoglobin equilibrium curve following hyperoxic exposure A 'right shift' of the oxygen dissociation curve along with a rise in RBC 2,3-DPG occurs following the administration of 19 ND to ambient mice or mice maintained in hyperoxia

*Discussion*

The mechanism by which androgenic steroids exert their effects on hematopoiesis has been of continued interest in view of their clinical application A moderate increase of the red cell mass has been observed in rodents following injection of these steroids [28, 38, 39, 44], but after prolonged administration a refractory state occurs [17], perhaps related to the dampening effect of the increased red cell mass on the production of ESF The effect of androgens on ESF production is well documented both in animals and in man [1, 14, 17, 26, 34] Recently 19 ND has been reported to be a strong erythropoietic stimulant in mice [20] and in man [3] The mechanism by which ESF production is stimulated by androgens is not clear Hypoxic exposure can stimulate ESF production even in a nephrectomized state [2, 14, 35, 43] Tissue hypoxia [41] or hypoxic exposure [40] may be compensated for by a 'right shift' of the oxygen dissociation curve [41] along with an increase of RBC 2,3 DPG Hyperoxia exerts a suppressive effect on the ESF level [22, 31] and has also been reported to suppress the RBC 2 3 DPG level [25]

*The relationship of androgen administration to the 'right shift' of the* oxygen hemoglobin equilibrium curve and the rise in RBC 2,3 DPG has been previously noted [18, 37] An effect of the androgen on the RBC metabolism resulting in an increased level of 2,3 DPG has been reported [37] However, it is somewhat difficult to reconcile this concept with the current observations A primary increase of RBC 2 3 DPG results in facilitated release of oxygen to the tissue therefore, the level of ESF may not be expected to rise This is not the case in the present studies The androgen 19 ND has increased both the level of ESF and the RBC 2,3 DPG and caused an increase in the $P_{50}$ ('right shift' of the oxygen hemoglobin dissociation curve) [18] When the mice were exposed to an increased oxygen tension the level of RBC 2,3 DPG decreased and the position of the

oxygen hemoglobin curve shifted to the left as expected [41] Androgen administration in hyperoxia resulted in an increase of the RBC 2 3 DPG and a shift of the oxygen hemoglobin equilibrium curve close to levels seen in the untreated ambient animal Thus it is not surprising that an associated increased level of ESF in animals exposed to hyperoxia and administered androgen was not observed Lack of detectable increase in plasma ESF following 19 ND administration in mice maintained in hyperoxia was not related to the presence of an inhibitor [9] as previously reported [12] Thus, it appears that the change in RBC 2 3 DPG following androgen administration is related to the level of tissue oxygenation rather than to the result of a primary effect on the RBC metabolism

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# A Second Case of Haemoglobin Belfast ($\beta$ 15 [A 12] Trp→Arg) Observed in a French Patient

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*Key Words* Haemoglobin Belfast Haemoglobinopathies Oxygen dissociation Unstable haemoglobins

*Abstract* Haemoglobin Belfast was initially observed in an Irish patient The second case found in a French patient suffering from a metastatic carcinoma, is here described Both observations are compared from clinical, haematological and biochemical points of view Functional and spectrophotometrical studies confirmed the slight abnormalities already described

Among the unstable haemoglobins, some give rise to severe haematological disorders, others are only discovered during a systematic survey or routine examination Haemoglobin Belfast ($\beta$ 15 [A 12] Trp → Arg) apparently belongs to this last group It was described in an Irish long-stay *psychiatric patient with polycystic kidneys [9] We report here the second* observation of this abnormal haemoglobin, incidentally found in a French patient suffering from a metastatic cancer

In all the known haemoglobins the residue at position A 12 is an hydrophobic one, usually tryptophan [6], acting as a spacer between helices A and E [12] As a consequence of the substitution of an arginine for this tryptophan, a destabilization of the globin structure and a slight increase of the oxygen affinity were described

## *Material and Methods*

Haematological studies were done following routine procedures.

Examination of the haemolysate by electrophoresis was carried out using acetate-cellulose plates (Titan III Helena Laboratories) in Tris EDTA-borate buffer, 8.8

Isoelectrofocusing on polyacrylamide gels was done according to the technique of DRYSDALE *et al* [7]

The isopropanol stability test was performed as described by CARREL and KAY [3]

The abnormal component was isolated and quantitated by DEAE Sephadex chromatography using a linear gradient of Tris HCl 0 05 M buffer from pH 8 0 to pH 7.3 The globin chains were separated by a Clegg's column [4] The tryptic digest of the amino-ethylated $\beta$ chain was analyzed by finger printing on silica gel thin layer Specific stainings were used for the identification of the spots [8]

The oxygen delivery function was studied on intact red blood cells (RBC) by the discontinuous spectrophotometric technique of BENESCH *et al* [2] in isotonic phosphate buffer, pH 7 15 [1] The intra-erythrocytic 2,3-diphosphoglycerate (2,3 DPG) was measured enzymatically following the formation of NADH at 340 nm (Sigma Technical Bulletin 35 UV) The lysate and the pure components were made free of organic phosphates by ion exchange chromatography [11], and the oxygen affinity was measured in 2,2 bis (hydroxymethyl) 2-2-2 -nitriloethanol (bis tris), 0 05 M buffer at 25 °C The pH dependence of log $P_{50}$ at 25 °C was measured in bis tris 0 05 M NaCl 0 1 M buffer below pH 7.5 and in Tris HCl 0 05 M, NaCl 0 1 M buffer above this pH value

Spectrophotometric studies were performed in 0 15 M phosphate buffer, pH 6.5, on a Beckman Acta III spectrophotometer

## *Case Report*

M F., a 53 year-old French man, was hospitalized for thoracic pain He had no previous pathological history and did not remember of any haemoglobin study He was the second child of unrelated French parents without known Celtic ancestry, both deceased Physical examination showed no abnormality apart from a painful tumefaction at the D 7 level and 9th right rib Routine blood count performed on a Coulter Counter model S showed an anaemia (see below), and a haemoglobin electrophoresis was routinely performed

A surgical biopsy of the 9th right rib showed a tumoral bone lesion. At histological examination, a diagnosis of metastatic carcinoma of poorly differentiated type was made. No primitive carcinoma was discovered after numerous radiological investigations including normal intravenous pyelogramm and renal arteriography

The patient was not yet treated when the haematological and biochemical studies were done

*Haematological studies* The blood film showed no abnormalities apart from hypochromia Haemoglobin 8.5 g/100 ml, red cells 3 18×10⁶/µl, packed cell volume 27 4%, mean corpuscular haemoglobin concentration 31 4%, mean corpuscular volume 85 µm³, reticulocyte count 45,000/µl without inclusion bodies on the smear

Serum iron level was 15 µg/100 ml with a total iron binding capacity of 135 µg/100 ml These results and the high erythrocyte sedimentation rate favour the diagnosis of inflammatory anaemia in relation with widespread carcinoma Serum bilirubin and haptoglobin were normal

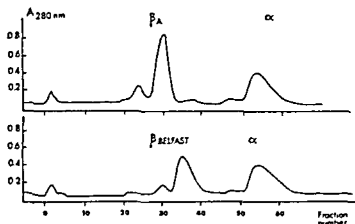



*Fig 1* Comparison between elution patterns of chain separation in Hb Belfast and Hb A

Numerous Heinz bodies were shown in the red cells after 24 h incubation at 37 °C and staining with methylviolet [5]

## *Haemoglobin Study*

An abnormal haemoglobin migrating like Hb S was observed during routine electrophoresis The solubility test was normal but an instability was observed within 30 min

The isoelectric point of the abnormal component, as determined by electrofocusing on polyacrylamide gel, was 7.23, the same as for Hb S and Hb D Punjab The percentage of the different haemoglobins was Hb $A_2$ 2 7%, Hb $A_1$ 68%, and Hb Belfast 29%

The elution pattern of the Clegg s column showed two abnormalities a β-chain eluted later than normal and a modified absorbance ratio at 280 nm between β- and α-chains. It was equal to 0 9 instead of 1 5 (fig 1)

The peptide map was the same as described by Kennedy *et al* [9] By ninhydrin staining there was no normal $\beta T_2$ and a faint $\beta T_3$ Three additional spots were seen (fig 2) The specific stainings showed the disappearance of β 15 tryptophan and a positive staining for arginine of two new peptides corresponding as described, to sequence β 9–15 and β 16–30 The third newly appeared spot was the dipeptide β 16–17

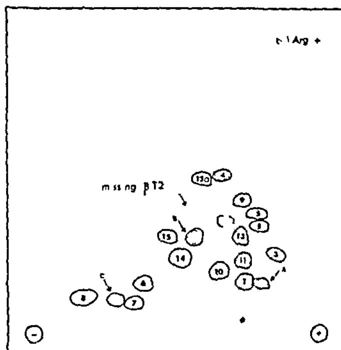


*Fig 2* The missing β T2 is in dotted lines Three additional peptides are shown by arrows, corresponding to new tryptic cleavages A = Residues 16 through 30 elongated β T3, without cleavage between residues 17 and 18 B = Residues 9 through 15 shortened β T2. C = Residues 16 and 17 cleavage at both Arg 15 and Lys 17

The oxygen dissociation curve of the patient's intact RBC was shifted to the right, the $P_{50}$ being 35 mm Hg (normal = 29 5 ± 1 0), but the intraerythrocytic 2,3-DPG was very high (24 μmol/g Hb instead of 13 ± 1) When compared to normal RBC containing the same amount of 2,3-DPG, the extent of the shift is in the normal range [10] On the stripped total lysate, the $P_{50}$ was 2 2 mm Hg This value is not significantly different from the normal

On the pure abnormal component, an increase of the oxygen affinity of about 10% was found In bis-tris 0 05 M, pH 7 15 buffer, the $P_{50}$ values were 1 88 mm Hg for Hb Belfast and 2 04 for Hb $A_1$ The same being observed at various pH values, the pH dependence curves of log $P_{50}$ were parallel, indicating an identical Bohr effect together with a higher oxygen affinity of the abnormal haemoglobin (fig 3) The co-operativity was always found normal The addition of known amounts of 2,3 DPG into the tonometer shifted the oxygen dissociation curve in an identical way for both haemoglobins

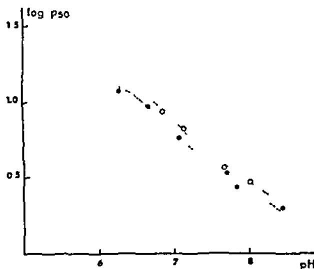



*Fig 3* pH dependence of the oxygen affinity ○ = Phosphate-free pure Hb A. ● = phosphate-free pure Hb Belfast.

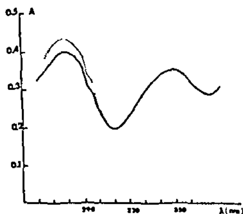



*Fig 4* Ultra violet absorption spectrum of oxyhaemoglobin Belfast (——) and oxyhaemoglobin A (-----). A difference is observed only in the ultra-violet part of the spectrum.

As expected from the disappearance of a tryptophan residue, spectrophotometrical abnormalities were observed When comparing the optical ultra-violet spectrum of the abnormal haemoglobin to normal, at the same concentration, a clear decrease of the absorbance was observed (fig 4)

## *Discussion*

This case is the second observation of Hb Belfast It was found in a patient of French origin (Burgundy) and no relation could be found with the previously described one In both cases an identical mutation may have occurred independently In the first description, the abnormal haemoglobin was associated with hereditary polycystic kidneys, and the haematological disorders were moderate In our patient, the kidneys were found normal and the severe anaemia observed had all the characteristics of a chronic inflammatory process, very likely due to the progress of the cancer No obvious symptoms of haemolysis could be correlated to the presence of an unstable haemoglobin The proportion of the abnormal component is close to what was described in the observation of KENNEDY *et al* [9] These authors showed that the ratio of synthesis was identical for Hb Belfast and Hb A in the reticulocytes, these results excluding the hypothesis that the iron deficiency found in both cases may explain a defective synthesis of the abnormal haemoglobin

From both observations, haemoglobin Belfast seems to be moderately unstable Nevertheless, some ambiguity remains due to the association of a severe disease which could explain non specific haematological disorders

The structural abnormality was easily identified by analytical fingerprint on silica gel thin-layer due to the identity with an earlier description Our findings concerning the oxygen equilibrium confirm the 'slight but significant' increase of the oxygen affinity previously described on the total lysate On the pure abnormal component, the oxygen affinity is clearly increased by about 10% Both this small difference in the oxygen delivery properties and the low percentage of Hb Belfast present in the cells explain why the alterations of the oxygen affinity are minimal in the total lysate and in the intact RBC.

*Acknowledgements* This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale and the Centre National de la Recherche Scientifique

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L. CAPRINO and E. C. ROSSI (ed.) **Platelet Aggregation and Drugs.** *1974 £ 5.80/US $ 15.25*

The proceedings of the 3rd Serono Symposium held in Rome contain 20 contributions distributed in 3 different chapters (1) Physiological and pathophysiological aspects of platelet aggregation, (2) Biochemical and pharmacological aspects of the platelet aggregation, and (3) Platelet aggregation control clinical potential Even though during the last years many symposia and conferences have been dedicated to platelets and mainly to their aggregation and its inhibition, these proceedings contain some original contributions or at least some original aspects of the recent developments Besides the more theoretical aspects of the platelet functions reviewed in the first and second chapters, the last chapter is devoted to practical problems which are of a more general interest to people in charge of patients and of their control This part of the book is certainly interesting but it solves less problems than it creates However, reading of *Platelet Aggregation and Drugs* can be recommended The papers are clearly written and easy to follow F. DUCKERT, *Basel*

M. W. STEWARD Immunchemie. Aus dem Englischen übersetzt von Dr. KLAUS ESSER Stuttgart 1975 VIII + 88 pp., 27 fig., 18 tab., DM 9.80 ISBN 3-437-20144-1

Auf kaum 80 Seiten Kleinformat ist es dem Autor gelungen, uns einen ausgezeichneten, klaren Überblick über die Immunchemie zu geben Neben der Struktur der Immunglobuline diskutiert er die physikochemischen Eigenschaften von Antigenen und die Grundlagen der Antigen-Antikörper-Reaktionen und der Komplementfixierung Ausser gesicherten Kenntnissen werden viele offene Fragen und Ausblicke erwähnt Eine ausgewählte Bibliographie, die bis 1974 reicht, hilft dem Leser den Zugang zur Originalliteratur zu finden Die «Immunchemie» kann allen immunologisch interessierten Biologen, Studenten und Ärzten wärmstens empfohlen werden T. L. VISCHER, *Genf*

Acta haemat 55 321-331 (1976)

# Hemopoietic Recovery in Bone Marrow of Lethally Irradiated Rats Following Parabiosis

I Granulopoiesis

With 1 color plate

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*Key Words* Bone marrow recovery after irradiation Granulopoiesis Hemopoiesis Irradiation Parabiosis

*Abstract* Aplasia was induced in rats by total body irradiation. Three days later, the animal was conjugated by aortic anastomoses with a healthy untreated litter mate 6 h after parabiosis, the bone marrow of irradiated animals contained some granulocytes showing RNA synthesis. At 18 h, many myelocytes and promyelocytes were present but no myeloblast was encountered These myeloid precursor cells showed active DNA synthesis but no mitoses, and no erythroblasts were observed at this time period At 24 h, mitoses of myeloblasts were found At 42–60 h, erythropoiesis was evident Chromosome analysis and investigations of cells of irradiated parabionts conjugated with partners having labeled cells, revealed that these newly formed myeloid and erythroid cells originated from the untreated parabiont. The mechanism of triggering myelopoiesis in the aplastic bone marrow by parabiosis is discussed

It is generally held that the various kinds of blood cells originate from a stem cell with a multidifferentiating potential [1] TILL and MCCULLOCH [2] reported the presence of bone marrow stem cells forming colonies in the spleen Other investigators reported that stem cells were found in circulating blood [3, 4] BECKER *et al* [5] and BARNES *et al* [6] reported that each cell colony originated from a single stem cell Independent *in vitro* studies by PLUZNIK and SACKS [7] and BRADLEY and METCALF [8] demonstrated colony formation of bone marrow and spleen cells in culture However, the morphologic identity of the stem cell is not clearly established

In the present investigation, stem cell activity was examined in rats after irradiation and parabiosis by light microscopy, by chromosome analysis, and by radioautography

## *Materials and Methods*

*Animals* Inbred adult Wistar rats weighing from 250 to 300 g were used (n = 262) They were divided into 5 groups 36 pairs of male male littermates for irradiation and parabiosis, 20 pairs of male female littermates for irradiation and parabiosis, 28 male irradiated (nonparabiosis) controls, 10 male nonirradiated (nonparabiosis) controls and 112 male aorta donors

*Irradiation* A single dose of total body irradiation at 1,000 rad, 200 kVP, 65 cm, was administered to one parabiont member (always male) prior to parabiosis Animals in the treated control group were exposed to the identical dose of irradiation

*Parabiosis* Three days after irradiation the irradiated member was joined in parabiosis with an untreated, healthy littermate Parabiosis was performed by the method of aortic anastomoses using donor aortas as extensions [9, 10] This procedure permitted the immediate and complete cross-circulation of blood between the

day that the prospective partner was subjected to irradiation) $^3$HTdr (specific activity, 5 Ci/mM) was injected intravenously, 0.3 mCi/100 g body weight once

*Chromosome analysis and mitotic index* At 3 h prior to sacrifice the male female parabionts were administered colchicine subcutaneously at 0 1 mg/100 g body weight Sex chromosome analysis by karyotype was performed with sedimented bone marrow cells using the method of OMURA [11] with 32–87 chromosome sets being analyzed in one sample The mitotic indices were obtained from irradiated animals by counting 2 000 sedimented nucleated cells

*Animal sacrifice* The animals were sacrificed by bloodletting The male male parabionts were sacrificed at 3, 6, 12, 18, 24 30, 36 42 and 48 h after parabiosis The male female parabionts were sacrificed at 18, 24, 36 48, 60, and 120 h after parabiosis 3–4 pairs of parabionts were sacrificed at each of these time periods Animals administered $^3$HTdr were sacrificed 18 h after parabiosis The irradiated control animals were sacrificed on day 3 4 5, and 6 following irradiation Animals found dead prior to sacrifice were discarded

*Blood cell and tissue preparation* At sacrifice about 10 ml of blood from the abdominal aorta was drawn heparinized and centrifuged at 1,300 rpm for 5 min, and the sedimented cells were smeared on slides and stained with May-Grünwald Giemsa From $^3$HTdr administered animals, peripheral smear specimens were mounted with sensitive emulsion exposed for 1 month, developed and stained with May Grünwald Giemsa

The bone marrow tissues were examined on the femurs of all the animals except for the donors of aorta A small amount of tissue was fixed in 10% formol for par

affin sectioning and stained by hematoxylin-eosin The remainder of the collected tissue was mixed with an equal volume of the rat serum and crushed gently in a glass homogenizer to free the hemopoietic cells from the tissue The cramped connective tissue was removed and the cell suspension was centrifuged at 1 000 rpm for 5 min the sedimented cells were smeared on slides and stained with May Grünwald-Giemsa. Some smears were stained with benzidine peroxidase reaction.

*Nucleic acid syntheses* in vitro The sedimented cells from each irradiated member of the male-male unions sacrificed at 6 12, and 18 h after parabiosis were divided into two parts one part was incubated with $^3$H uridine ($^3$HUr) at 10 $\mu$Ci/ml and 1 mM of cold thymidine for 40 min, and the other part was incubated with $^3$HTdr at 1 $\mu$Ci ml for 40 min The incubation medium was 3 ml of Hanks solution with an equal volume of rat serum. After incubation, the cells were sedimented and washed in 10 ml of Hanks solution, smeared on slides, mounted with sensitive emulsion (Sakura NRM2) exposed for 3 weeks, and developed and stained with May Grünwald Giemsa

*Morphologic studies* were conducted on samples from all animals. Cell classification studies were performed on smears from all groups, except for the male female parabionts

## *Results*

In irradiated controls the bone marrow was aplastic 3 days after irradiation Examination of bone marrow smears indicated that the nucleated cells were plasma cells and fibroblast like cells, or reticulum cells In some exceptional samples atypical myeloid and lymphoid cells were occasionally encountered Mitosis was found in about 0 3% of cells and these cells were probably interstitial cells and plasma cells as indicated by their morphologic feature Most animals died by 5 days after irradiation Those surviving for 6 or 7 days following irradiation showed neither myelocytes, mature granulocytes nor lymphoid cells indicating that the myeloid precursor cells found occasionally at 3 days after irradiation were destined to die and were without replicating potency

*Recovery of hemopoiesis by parabiosis* Irradiated animals conjugated by parabiosis showed almost complete hemopoietic recovery at 5 days following parabiosis Bone marrow tissue sections at this time period showed active myelopoiesis and erythropoiesis One day after parabiosis myeloid precursor cells with mitoses were present but no erythroblast was found Two days after parabiosis, distinct erythropoiesis with active myelopoiesis was observed.

*The mitotic index* of bone marrow cells of irradiated parabionts was low 18 h after parabiosis compared to the irradiated control group However, from day 2 following parabiosis the mitotic index increased and at

*Table I* Mitotic indices and number of female mitotic cells from the bone marrow of irradiated male rats joined to untreated females by parabiosis

| Hours after parabiosis | Number of pairs | Mitotic index[1], % (mean ± SD) | Number of mitotic cells | | |
|---|---|---|---|---|---|
| | | | female cell number | cells counted | female cells/total cells, % |
| 18 | 3 | 0 28±0 12 | 2 | 49 | 4 1 |
| | | | 2 | 55 | 3 6 |
| | | | 1 | 68 | 1 5 |
| 24 | 4 | 0 64±0 15 | 20 | 57 | 35 1 |
| | | | 13 | 50 | 26 0 |
| | | | 20 | 72 | 27 8 |
| | | | 18 | 59 | 30 5 |
| 36 | 3 | 0 95±0 10 | 30 | 59 | 50 8 |
| | | | 44 | 88 | 50 0 |
| | | | 22 | 43 | 51 2 |
| 48 | 3 | 1 20±0 15 | 30 | 59 | 50 8 |
| | | | 32 | 65 | 49 2 |
| | | | 34 | 71 | 47 9 |
| 60 | 4 | 2 16±2 70 | 41 | 52 | 78 8 |
| | | | 55 | 76 | 72 4 |
| | | | 16 | 32 | 50 0 |
| | | | 40 | 72 | 55 6 |
| 120 | 3 | 9 46±0 44 | 81 | 84 | 96 4 |
| | | | 57 | 60 | 95 0 |
| | | | 80 | 87 | 92 0 |
| Irradiated control | 3 | 0 32±0 06 | – | – | – |
| Untreated partners | 3 | 5 50±0 70 | – | – | – |

[1] The mitotic index was determined in each animal by counting 2,000 cells

day 5 after parabiosis the value exceeded the level of the healthy untreated partner (table I)

*Chromosome analysis* on bone marrow cells of irradiated males conjugated with females revealed that a few female cells began mitosis in the male aplastic bone marrow 18 h after parabiosis, and these cells entered into active mitoses 24 h after parabiosis Thereafter, the percentage of fe-

*Table II* Classification of cells collected from irradiated bone marrow of male-male parabionts (mean values from 3 or 4 pairs)

| Cell groups | Healthy controls | Irradiated controls | Hours after parabiosis | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3 | 6 | 12 | 18 | 24 | 30 | 36 | 42 | 48 |
| Myeloid cells | | | | | | | | | | | |
| Mature cells | 26.2 | 6.6 | 19.8 | 28.0 | 27.5 | 32.9 | 26.7 | 12.5 | 13.5 | 11.2 | 21.7 |
| Myelocytes | 4.0 | 0.1 | 0 | 0 | 1.7 | 3.8 | 1.8 | 0.5 | 2.0 | 3.2 | 3.0 |
| Promyelocytes | 1.8 | 0 | 0 | 0 | 0 | 4.2 | 3.5 | 3.0 | 1.5 | 2.5 | 2.0 |
| Myeloblasts | 0.4 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0.5 | 1.0 | 1.2 | 0.5 |
| Lymphoid cells | 26.5 | 0.6 | 29.0 | 24.5 | 36.3 | 31.2 | 31.5 | 50.0 | 45.0 | 15.2 | 6.0 |
| Monocytes | 2.0 | 0.9 | 0.2 | 2.0 | 1.5 | 1.3 | 1.0 | 0.3 | 2.0 | 1.5 | 0.6 |
| Plasma cells | 0.8 | 72.9 | 29.4 | 21.5 | 28.2 | 22.9 | 32.2 | 24.0 | 24.0 | 38.9 | 8.5 |
| Reticulum cells | 5.0 | 15.9 | 21.5 | 22.5 | 4.3 | 3.5 | 2.5 | 9.0 | 10.5 | 17.5 | 1.5 |
| Megakaryocytes | 0.2 | 3.0 | 0.1 | 1.5 | 0.5 | 0.2 | 0.6 | 0.2 | 0.5 | 0.1 | 0.2 |
| Erythroblasts | 33.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.7 | 56.0 |

male mitotic cells increased to 50% after 48 h and to 95% after 120 h, indicating that hemopoietic recovery was made by cells originated from the healthy female partner (table I)

*Morphologic observations* on bone marrow cells in irradiated males conjugated with healthy males revealed that 3 h after parabiosis dramatic accumulations of granulocytes and lymphoid cells were found in bone marrow sections and smears These cell accumulations were not encountered before parabiosis Granulocytes constituted about 20% with lymphoid cells and plasma cells each constituted about 30% of nucleated cells, monocytes constituted less than 1% No erythroblasts were encountered Almost all granulocytes were mature and some metamyelocytes were present but no myelocytes, promyelocytes or myeloblasts were found (table II) Small and large lymphoid cells were found in about equal numbers

At 6 h after parabiosis, the frequency of the bone marrow cells was about the same as that seen 3 h after parabiosis. *However, some large* granulocytes appeared, 12–15 μm in diameter with a segmented or ring-form nucleus and these cells showed a slightly basophilic cytoplasm (fig 1a)

At 12 h after parabiosis the cellularity of the bone marrow increased.

The distinctive feature of this period was the appearance of some myelocytes (table II) These cells had a thick ring-form or penetrated nucleus, fine chromatin nets, moderately basophilic cytoplasm, and most cells contained many azurophilic granules (fig 1b) The cytoplasm indicated a strong positive peroxidase reaction (fig 1g) The percentage of monocytes remained small

At 18 h after parabiosis many younger myeloid precursor cells appeared (table II) Most of them were myelocytes and promyelocytes, though a few myeloblasts were found by careful observation The characteristic of this stage was the appearance of promyelocytes having a solid nucleus They were large in size, had a basophilic cytoplasm with many azurophilic granules (fig 1c) and gave a strong peroxidase reaction by which they were distinguished from monocytes

At 24 h after parabiosis the cellularity of the bone marrow continued to increase and some young myeloblasts appeared These cells showed a deeply basophilic cytoplasm and some azurophilic granules (fig 1d) Mitoses of myeloid cells were occasionally observed Granulocytic cells constituted about 30% of the total cell population, and the myeloid precursor cells occupied about 17% of the total granulocytic cells (table II)

Lymphoid cells occupied about 30% of the total nucleated cells during the period from 3 to 24 h after parabiosis During this interval plasma cells constituted about 30% of cells compared to the irradiated control value of 73%, and reticulum cells decreased from 21 5 to 2 5% Monocytes showed little change during this period (table II) The morphologic investigation failed to reveal cells suggestive of transition from lymphoid or monocytoid cells to myelocytes, promyelocytes, or myeloblasts

In the period 30 and 36 h after parabiosis, distinct myelopoiesis was present in the bone marrow tissues In smears, myeloid precursor cells increased to about 25% of the granulocytic cell population The percentage of lymphoid cells increased markedly but no erythroblasts were encountered (table II)

At 42 and 48 h after parabiosis, the cellularity of the bone marrow tissue increased still further Some younger erythroid precursor cells appeared in smears from some animals at 42 h after parabiosis An abrupt increase of erythroblasts was found at 48 h after parabiosis in irradiated animals Erythroblasts constituted more than 50% of the total nucleated cells and a marked decrease of lymphoid cells was present (table II)

*Radioautography* of bone marrow cells of the irradiated member collected 6 h after parabiosis and incubated with $^3$HUr *in vitro*, revealed



*Fig 1* Cells found in the aplastic bone marrow of an irradiated rat after parabiosis with a healthy litter mate *a* Metamyelocyte found 6 h after parabiosis *b* Myelocyte found 12 h after parabiosis *c* Promyelocyte found 18 h after parabiosis *d* Two myeloblasts found 24 h after parabiosis *e* Uptake of $^{3}$HUr by mature granulocytes 6 h after parabiosis *f* Uptake of $^{3}$HTdr by a myelocyte 12 h after parabiosis *g* Peroxidase reaction of mature granulocytes myelocytes and promyelocytes

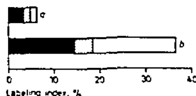



*Fig 2* Mean labeling index of bone marrow cells by ³HTdr of irradiated (a) and nonirradiated (b) parabionts 18 h after parabiosis The labeling index was determined in each animal by counting 5 000 bone marrow cells. Dark area = myeloid cells, hatched area = lymphoid cells, open area = labeled cells other than myeloid and lymphoid cells

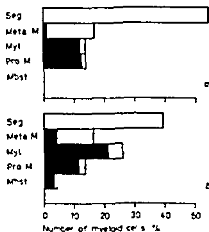



*Fig 3* ³HTdr labeling index of myeloid cells of irradiated (a) and nonirradiated (b) parabionts at 18 h after parabiosis. Labeling index was obtained by counting 2,000 myeloid cells from bone marrow in each animal Seg = mature granulocytes, Meta M = metamyelocyte, Myl = myelocyte, Pro M = promyelocyte, Mbst = myeloblast open area = nonlabeled cells dark area = labeled cells.

RNA synthesis in large-sized granulocytes and metamyelocytes (fig 1e) These cells showed nuclear RNA synthesis without DNA synthesis Active RNA and DNA syntheses were observed in myelocytes found in the aplastic bone marrow 12 h after parabiosis (fig 1f) and also in promyelocytes at 18 h after parabiosis

In the *in vitro* study of DNA synthesis at 18 h after parabiosis, the labeling index of bone marrow cells was 6.3% and that of myeloid cell was

3.7%. In untreated parabionts labeling index of bone marrow cells was about 36% and that of myeloid cells was 13.7% (fig. 2). In irradiated animals, nearly 95% of all the precursor cells, myeloblasts, promyelocytes, and myelocytes, were found to be labeled (fig. 3a), while in those of the nonirradiated partner only 70% of myeloid precursors were labeled (fig. 3b). The labeling index of lymphoid cells was rather low compared to myeloid cells (fig. 2), although some large sized lymphoid cells were highly labeled. A similar tendency was observed in the labeling of lymphoid cells of the untreated partner.

*In irradiated animals conjugated with partners having mature granulocytes labeled with ³HTdr* and sacrificed 18 h after parabiosis, the irradiated bone marrow contained labeled myeloid precursor cells and lymphoid cells, indicating that these cells migrated from the healthy partner.

*Observations of peripheral blood smears* at various time periods after parabiosis revealed no leukemoid reaction suggestive of a massive transfer of myeloid precursor cells from the healthy member. No myeloblasts or promyelocytes were found.

## *Discussion*

Aplastic bone marrow of irradiated rats started granulopoiesis 24 h after parabiosis with a healthy littermate. Myelocytes, promyelocytes, and a few myeloblasts were observed 18 h after parabiosis, but mitosis was hardly encountered in these cells. At 24 h after parabiosis some myeloblasts in active mitosis appeared. Erythropoiesis appeared 42–48 h after parabiosis. Investigations with labeled cells and by chromosome analysis revealed that the cells participating in hemopoiesis in the irradiated bone marrow originated from the healthy partner.

*The hemopoietic recovery of the aplastic bone marrow may have been* directed by stem cells in circulating blood. In observations of colony-forming units (CFUs), McCULLOCH and TILL [12] noted that the cell number in any one colony showed an exponential increase with a 24 hour lag phase after transplantation, suggesting that mitosis in CFUs begins at 24 h after transplantation. This 24-hour lag phase is consistent with the granulopoiesis in the irradiated animal joined with a healthy partner. However, the precedency by erythropoiesis followed by granulopoiesis in CFUs [1] is inconsistent with the case.

The hemopoietic response becomes evident by the initial appearance of undifferentiated cells or the youngest precursor cells, followed by more

differentiated cells with mitosis In the present experiment, the erythropoiesis seen after parabiosis is consistent with the contemporary concept of hemopoiesis involving the stem cell of CFUs The erythropoiesis was triggered 42–48 h after parabiosis with the sudden appearance of the youngest precursor cells, followed by an abrupt increase of more differentiated erythroblasts

In myelopoiesis, however, the appearance of the youngest precursor cells was preceded by the appearance of more differentiated precursor cells This sequence differs from the process in erythropoiesis and is inconsistent with the general concept of hemopoiesis originating from the stem cell However, the participation of the stem cell or the transformation of lymphoid [13] or monocytic cells [14, 15] to myeloid precursor cells and the direct transformation of the stem cell to myeloblasts from its $G_0$ stage without differentiating mitosis should be considered [16]

In the present study a number of lymphoid cells appeared in the aplastic bone marrow after parabiosis, and large lymphocytes increased with time However, no cell was encountered that suggested a transformation of these cells to myeloid precursor cells, i e, myelocyte, promyelocyte, and myeloblast The myeloid precursor cells were clearly distinguished from lymphoid cells by a strong positive peroxidase reaction

TYLER and EVERETT [15] reported that in parabiotic rats, whose partner had $^3$HTdr labeled cells by treating with $^3$HTdr injections and temporary arrest of cross-circulation between 2 animals, some $^3$HTdr labeled blast cells appeared in the bone marrow of the untreated animal 24 h after the last injection of $^3$HTdr The blast cells demonstrated in their paper were very similar to those classified as myeloblasts and promyelocytes in this paper They supposed that monocytoid cells may give rise to these blast cells but we could not find any morphologic evidence to the direct transition of monocyte to these myeloid precursor cells They had distinct promyelocyte granules and gave strong positive peroxidase reaction, while monocyte had only faint azurophil granules and were slight or negative in peroxidase reaction

Another possibility is the direct transfer of myeloid precursor cells from the healthy partner to the irradiated animal The buffy coat smears of peripheral blood were examined carefully but neither promyelocytes nor myeloblasts were detected after parabiosis Tissue sections of liver, spleen and lymph nodes were observed but neither changes suggestive of leukemoid reaction nor accumulations of myeloid precursor cells were detected [17] The leukemoid reaction is generally accompanied by the mo-

bilization of erythroblasts, but in the present experiment erythroid cells were hardly encountered in smears of circulating blood Furthermore, no erythroblast was found in the bone marrow of irradiated animals within 36 h after parabiosis Almost all myeloid precursor cells in irradiated animals were at the S phase, while in the bone marrow of healthy partners many precursor cells were at the $G_1$ and $G_2$ stages These observations are contrary to the direct transfer of the myeloid precursor cells from the healthy partner to the irradiated animal

The blast formation of mature granulocytes has not been reported, but a possible explanation for the phenomenon observed in the present experiment appears to be the dedifferentiation of the granulocytes to myeloid precursor cells *in situ* and their participation in a new myelopoiesis in the aplastic bone marrow Evidence for this possibility includes accumulation of granulocytes in the irradiated bone marrow 3 h after parabiosis, RNA synthesis of some granulocytes at 6 h, appearance of myelocytes at 12 h, presence of promyelocytes with active RNA and DNA syntheses but without mitosis at 18 h, and the appearance of some myeloblasts with active mitosis at 24 h after parabiosis

As reported in *in vitro* studies, the metamyelocyte may transform to myelocyte with a change in nuclear form [18] Myelocytes seem to have a self-renewing activity [19] Accordingly, metamyelocytes may induce granulopoiesis In the present experiment, however, some cells having segmented nuclei showed RNA synthesis and this was followed by the appearance of myelocytes in the irradiated bone marrow Therefore, it is not unconceivable that some granulocytes may rejuvenate to myelocytes

In the present experiment, mitosis first appeared at the stage where myeloblasts were observed, and was hardly observed at the time when myelocytes and promyelocytes appeared Therefore it appears that myelocytes have a self renewing activity but after dedifferentiation to myeloblast Thus, in bone marrow some granulocyte may have the potency to rejuvenate to blast cell like as lymphocytes and participate in granulopoiesis under a condition such as severe hemopoietic tissue damage by lethal irradiation, although confirmation will require further investigations

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# Reconstitution Capacity of Bone Marrow Cells from Nude Mice in Radiation Chimeras

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*Key Words* Bone marrow reconstitution · Nude mice · Radiation chimeras · Transplantation

*Abstract* Lethally irradiated mice were reconstituted with bone marrow cells from 'nude' or normal mice It was found that the radioprotective capacity of bone marrow cells from normal and thymusless 'nude' mice is similar and that the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar Bone marrow cells from nude mice in spite of the lack of the thymus develop into hematopoietic cells and contain also T precursor cells

Stem cells from the bone marrow are able to develop into cells of the erythroid, granulocytic and lymphoid series following transplantation into recipients receiving potentially lethal dose X-irradiation to result in radiation chimeras [4, 6, 8, 11] It is not clear how this development is controlled One of the controlling mechanisms postulated is the thymus The role of thymus in the development of the lymphoid system is well documented [2] and its role in hematopoiesis has also been suggested [3, 10] ZIPORI and TRAININ [17] have reported that the radioprotective capacity of the bone marrow cells from neonatally thymectomized mice was greatly impaired and that bone marrow cells from nude mice (nu/nu) which lack the thymus congenitally, only occasionally exhibit radioprotection [17] This paper describes experiments showing that (a) the radioprotective capacity of bone marrow cells from normal and nude mice is similar and that (b) the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar

## *Material and Methods*

Normal C3H and C57Bl 6 mice and homozygous nu nu mice backcrossed (4th–5th backcross) with C3H and C57Bl 6 backgrounds were used (purchased from Gl Bomholtgard Ltd., Ry, Denmark) Donor and recipient animals were 8–10 weeks old Bone marrow cells were prepared in cold from tibiae and femora in Eagle s minimal essential medium supplemented with 10% fetal calf serum. Donor cells were pooled and injected intravenously at the indicated dosages within 2–3 h following irradiation Cell doses per mouse ranging from $1\times10^7$ to $1\times10^5$ from normal and nude mice were tested for their ability to save lethally irradiated recipients. 10 to 20 mice were used for each group Animals were kept on antibiotics (100 mg Neomycin and 10 mg Polymyxin/liter added in drinking water) for 2 weeks and observed daily for mortality for 3–4 months.

The recipients were prepared for the bone marrow graft by exposure to 850 r of total body irradiation given at a rate of 35 r/min and a distance of 25 cm by a Philips RT 305 X ray machine (300 kV, 10 mA, total filtration 2 7 mm (Cu))

## *Results*

Figure 1 shows that the mortality rates of lethally irradiated C3H mice reconstituted with varying doses of bone marrow from nude C3H mice and from normal C3H mice were similar With $1\times10^7$ bone marrow cells there was no mortality With $3\times10^5$ bone marrow cells the mortality was 23%, whereas with $1\times10^5$ cells it was 50%. All control mice which received no bone marrow cells died within 2 weeks Figure 2 shows the mortality curve with C57Bl/6 mice With bone marrow cell doses of $1\times10^7$, $3\times10^4$ and $1\times10^5$ cells, mortality amounted to 21, 38 and 61%, respectively, for those reconstituted with normal C57BL6 bone marrow cells, and to 30, 16 and 84% for those reconstituted with nude C57BL6 bone marrow cells All controls which were only irradiated died within 2 weeks The mortality rates of mice restored with equal cell doses from either nude or normal donors were not significantly different (Fisher s exact test) The results with both mouse strains show the equivalent potency of bone marrow cells from nude and normal mice to rescue irradiated mice by restoring their hematopoiesis

To test the reconstitution of the immunological capacity of the surviving mice, sheep red blood cells (SRBC) were chosen as the antigen because the immune response to this antigen in the normal [13] and nude [15] mice is well documented It is classified as a T-cell (thymus cell) dependent antigen The immune response in the nudes is primarily of the IgM type A single injection of 0.5 ml of 10% sheep red cells was given

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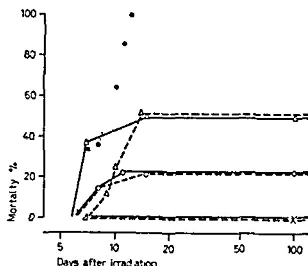



*Fig 1* Mortality of 850 r irradiated C3H mice transplanted with varying doses of bone marrow cells from normal (———) and nude (- - -) C3H mice ● = Controls, no cells Δ = $1 \times 10^5$ cells ○ = $3 \times 10^5$ cells × = $1 \times 10^7$ cells

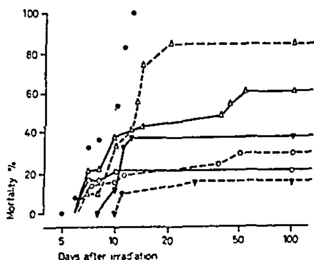



*Fig 2* Mortality of 850 r irradiated C57Bl/6 mice transplanted with varying doses of bone marrow cells from normal (———) and nude (- - -) C57Bl/6 mice ● = Controls, no cells. Δ = $1 \times 10^5$ cells. ▼ = $3 \times 10^6$ cells ○ = $1 \times 10^7$ cells

*Table I* Immune response to SRBC in radiation chimeras produced by normal and nude C3H bone marrow cells transplanted into lethally irradiated C3H mice

| Bone marrow cells transplanted | | PFC/10⁶ spleen cells | |
|---|---|---|---|
| donor | cell dose per mouse | direct | indirect |
| nu/nu | $3 \times 10^7$ | 154±42 | 892±284 |
| | $1 \times 10^7$ | 83±19 | 615±216 |
| Normal | $3 \times 10^7$ | 139±30 | 795± 90 |
| | $1 \times 10^7$ | 119±37 | 577±122 |

Hemolytic plaque forming spleen cells (PFC) were assayed 7 days after intravenous injection of SRBC and 4 months after bone marrow transplantation. Mean values ± SE for groups of 4–6 mice are given. The respective differences of the PFCs after nu/nu and normal bone marrow cells are not statistically significant

intravenously or intraperitoneally to the radiation chimeras 2–4 months after bone marrow transplantation The spleen cells were assayed 7 days later for cells producing IgM antibodies (direct plaques) and IgG antibodies (indirect plaques) according to the CUNNINGHAM and SZENBERG [1] modification of the hemolytic plaque assay [5] Table I shows that the number of cells producing antibodies against sheep red cells in irradiated C3H mice reconstituted with nude and normal C3H bone marrow cells 4 months earlier was similar There were both IgM and IgG antibody producers, with more of the latter type, similar to that found in the normal mice This means that their immunological capacity, at least to this antigen was probably fully reconstituted Table II shows a similar result from irradiated C57Bl/6 mice 2 months after bone marrow transplantation

## *Discussion*

The results reported here imply that there is no defect exhibited by the bone marrow cell populations in the nude mice with radioprotection and immune reactivity as indices in spite of the lack of the thymus These cells transplanted into lethally irradiated mice were able to develop not only into hematopoietic cells as shown by their ability to save lethally irradiated mice, but also into lymphoid cells including thymus cells as shown by the unimpaired ability to produce both IgM and IgG responses to a 'thymus-dependent' antigen One could always raise the question

*Table II* Immune response to SRBC in radiation chimeras produced by normal and nude C57Bl/6 bone marrow cells transplanted into lethally irradiated C57Bl 6 mice

| Bone marrow cells transplanted | | PFC/$10^6$ spleen cells | |
|---|---|---|---|
| *donor* | *cell dose per mouse* | *direct* | *indirect* |
| nu/nu | $1 \times 10^7$ | $475 \pm 14$ | >2 000 |
| Normal | $1 \times 10^7$ | $533 \pm 81$ | ~2 000 |

*PFC* were assayed 7 days after intraperitoneal injection of SRBC and 2 months after bone marrow transplantation Mean values ± SE for groups of 5 mice are given The differences are not statistically significant

whether these results are real in that the nude bone marrow cells did indeed develop normally as normal bone marrow cells following transplantation into lethally irradiated recipients or are deceptive in that the irradiated hosts recovered from the irradiation effects The second possibility is rendered unlikely by experiments showing that the hematopoietic and lymphoid cells in radiation chimeras produced in this way are predominantly of the donor instead of the recipient type [6, 9, 12] If this is the case, the T-cells in the mice reconstituted with nude bone marrow cells would have to come from these transplanted cells In other words, there are T-precursor cells in the nude bone marrow as observed by other investigators [9, 14] LOOR and KINDRED [7] have also come to the same conclusion by studying the T-cell membrane markers following grafting neonatal AKR thymus (TL , θ AKR) into Balb/C nude mice (TL2, θ C3H) Experiments are under way using allotype markers to study the possible switch-over of the recipient allotype to donor allotype in these radiation chimeras reconstituted with bone marrow cells from the nude mice

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# Characterization of Blast Cells in Acute Nonlymphoid Leukemias by Consecutive Cytochemical Reactions

With 1 color plate

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*Key Words* Acute leukemia Blast cells Combined cytochemical stainings Cytochemistry Esterases Leukemia

*Abstract* Blast cells of 12 acute nonlymphoid leukemias have been investigated by consecutive application on the same preparation of three cytochemical reactions α naphthylacetate esterase AS D choroacetate esterase and PAS A fourth reaction (α naphthylbutyrate esterase) was added in some preparations for a more detailed definition of the monocytoid oriented cells The cytochemical techniques were further combined with NaF inhibition and with the cytobacterial test for muramidase The combined cytochemical approach allowed the identification of most of the blast cells, according to an empirically determined positivity to one or more reactions and proved to be particularly valuable in classifying the cells after chemotherapy

The variable responsiveness of acute leukemias to chemotherapy has supported further attempts to classify different cytological variants with particular prognostic properties In this regard several approaches have been made recently with reference to the pictures in Romanovsky preparations [18, 19], as well as to immunocytological properties [1, 6, 17], cytochemical features [2, 11, 20], ultrastructural aspects [3], and some functional characteristics [7, 23]

While the acute lymphoblastic leukemias have been recently characterized more accurately on the basis of the immunocytological markers much more difficulties have occurred in the definition of the acute nonlymphoid leukemias which apparently show a minor homogeneity Two major problems have been encountered in the classification of the blast cells in the nonlymphoid acute leukemias (1) the search for suitable markers of the myeloid cell series, (2) the possibility to identify the differen-

tiation of the myeloid cells, with particular reference to the monocytoid elements [26]. These difficulties are explaining the requirement of additional tests in order to achieve a more complete evaluation of the activity of the blastic elements [7, 12, 23].

Since most of the therapeutic managements are performed accordingly to the criteria of the bone marrow composition and the peripheral blood picture, the morphological approach appears still to be the most valuable tool for diagnostic purposes [10].

A series of cytochemical and cytofunctional tests consecutively applied on the same slide has provided the possibility to classify most of the blast cells and of their differentiation. This consecutive staining procedure proved to be particularly useful in our hands for the characterization of the blastic population during the transformation of the chronic myeloid leukemia [5].

## *Material and Methods*

12 patients of both sexes, aged between 18 and 74 years, with acute nonlymphoid leukemias were examined before treatment and during the course of the illness for a follow up of the blastic cell population. For this purpose three cytochemical reactions were consecutively performed on the blood smears: α naphthylacetate esterase (α NAE) [15], AS-D-chloroacetate esterase (AS-D-Ch AE) [13] and PAS. A fourth reaction, the α naphthylbutyrate esterase (α NBE) [14], was added. The use of the α NBE provided more reliability to the definition of the monocytoid component of the population, since the substrate employed in this reaction has been ac

| Reactions | | Cells |
|---|---|---|
| AS-D Ch AE<br>AS-D Ch AE + PAS (granular) | | → myeloblasts |
| AS-D-Ch AE + α NAE (or α NBE) | | → promonocytes |
| α NAE | + PAS (strong and coarse irregular positivity) | → megakaryoblasts |
| | + PAS (diffuse or fine granular<br>+ NAE (inhibition)<br>+ lysozyme (production)<br>α NBE (substrate substitution) | monoblasts, monocytes |

*Fig. 1.* Identification of blast cells by combined cytochemical reactions.

*Table I* Percentage of blast cells with positive reaction

| | Case No | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Promyelocytes, myeloblasts | | | | | | | | | | | | |
| AS-D-Ch-AE | | | | | | | | | | | | |
| AS-D-Ch-AE+PAS | 91 | 63 | 60 | 7 | 79 | 40 | 62 | 42 | 74 | 57 | 41 | 68 |
| Promonocytes | | | | | | | | | | | | |
| AS-D-Ch-AE+α-NAE | | | | | | | | | | | | |
| AS-D Ch-AE+α-NBE | 5 | 14 | 19 | – | 8 | 23 | 15 | 11 | 5 | 8 | 16 | 6 |
| Megakaryoblasts | | | | | | | | | | | | |
| α-NAE | | | | | | | | | | | | |
| α-NAE+PAS | – | – | 1 | – | 2 | – | – | 1 | – | 3 | – | – |
| Monoblasts, monocytes | | | | | | | | | | | | |
| α-NBE | | | | | | | | | | | | |
| α-NBE+PAS | 4 | 23 | 20 | 11 | 11 | 37 | 21 | 46 | 21 | 32 | 43 | 24 |
| No reaction | – | – | – | 82 | – | – | 2 | – | – | – | – | – |

tually demonstrated to be strongly specific for the monocytic series [14] The combined reactions allowed the differentiation of most blast cells, according to an empirically determined positivity to one or more reactions (fig 1)

500 cells with blastic features were scored on each preparation and a percent distribution, according to the previously defined cytochemical characteristics, was reported

The definition of the mononuclear elements (monocytic) has been further investigated by means of the cytobacterial test for the muramidase production [25] and the inhibition test with (NaF) [22], applied together with the multiple cytochemical stainings on the same slide

Some preparations were finally decolorized in xylene with removal of the cytochemical staining, and subsequently restained with May-Grünwald Giemsa in order to obtain a comparison between the usual panoptic features of the cells and the cytochemical properties demonstrated by the consecutive stainings

## *Results*

The results of the distribution of the blast cells according to the combined cytochemical features are listed in table I. Apart from an unclassifiable case only a small proportion of the blastic cells was not identified Most of the cases were mixed forms with a variable participation of granuloblastic and monocytoid cells. Pure myeloblastic forms (more than 80%

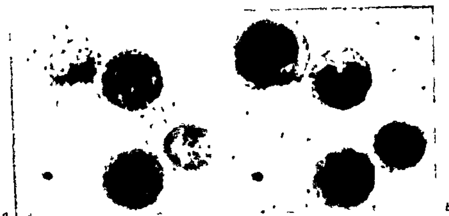



*Fig. 2.* *a* Combined cytochemical reactions for AS-D-Ch-AE and α-NBE. Myeloblastic elements present the cytoplasm filled with dark granules (fast violet LB salt), while the monocytoid cells show coarser granules (fast garnet). *b* Same picture after decoloration and restaining with May-Grünwald-Giemsa. Two cells with similar shape and indented nuclei appear to be differently classified on the basis of the combined cytochemical procedures (case No. 8). ×670.

of the elements) represented only a minor fraction of our cases 1 case out of 12 was unclassifiable because of the lack of all the markers in most of the cells (case No 4) This case possibly could be interpreted as a poorly differentiated leukemia

Monocytoid elements were clearly identified with the reaction for the α-NBE The usefulness of this reaction in association with the AS-D Ch-AE could be further evaluated by rewieving the cells after elution of the cytochemical stainings and restaining with May-Grunwald-Giemsa In fact mononuclear cells with indented nuclei which appear quite similar in Romanovsky preparations, could be easily identified as belonging to different lines (granulocytic and monocytic, respectively) in the cytochemically treated smears (fig 2, 3)

The introduction of the cytobacterial test in association with the cytochemical reactions allowed a finer distinction of the monocytoid elements with regard to their degree of maturation Monoblastic cells were characterized by a weak α-NAE activity (eventually associated with a fine granular PAS positivity) and by a poor muramidase secreting activity, in contrast with the more nature monocytic cells which showed usually a pronounced muramidase production (fig 4) Inhibition with NaF could further confirm the nature of these elements

An interesting observation was the appearance in a few cases of elements with the features of megakaryoblasts well defined by a strongly positive α-NAE reaction (sometimes associated with coarse PAS-positive granules surrounding the cytoplasm) and by an almost completely negative reaction for the α-NBE These elements, which are very common in the blastic crisis of the chronic myeloid leukemia were demonstrable only in a minority of the cases of acute leukemia and in a small percentage

## *Discussion*

On the basis of the cytochemical distribution of the blast cells in our cases of acute nonlymphoid leukemias the following types are likely to be recognized myeloblastic, promyelocytic, promonocytic, myelomonocytic, monocytic, and unclassifiable This distinction is in agreement with the functional differentiation of the blast cells [7–9] Particularly, similar results have been obtained by BENNETT and REED [2] utilizing an empirically determined positivity to a series of different reactions (peroxidase, PAS NASDA and NASDA + F)

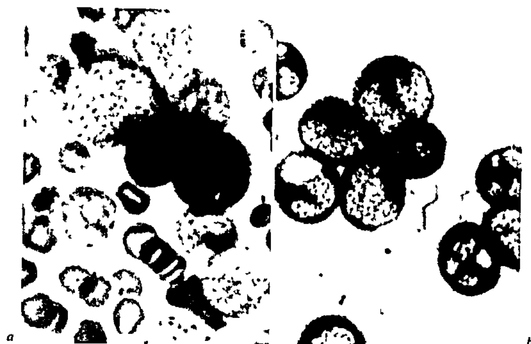


*Fig 3* *a* Combined AS D Ch AE and *a* NBE (case No 12) *b* Combined AS D Ch AE + *a* NBE + PAS (case No 12) Monocytoid cells may show, besides the positive reaction for the *a* NBE, several fine PAS positive granules scattered over the cytoplasm ×1,000

In our opinion the most outstanding aspect of the consecutive application of the cytochemical reactions seems to be represented by a more detailed definition of the monocytoid-oriented cells and possibly, by the differentiation between the monoblastic and the more mature monocytic elements The possibility to identify the megakaryoblastic elements may be another important point, since small megakaryoblasts have been recognized only by means of multiple combined approaches, including the ultrastructural procedures [16, 24] in several conditions of preleukemia and in a variety of myeloproliferative disorders [16] With the combined cytochemical stainings they were easily recognized in a significant number in the blastic crisis of various cases of chronic myeloid leukemia where they may represent a great part of the blast cell population [4] In acute non-lymphoid leukemias the megakaryoblasts did not represent a significant component of the blast cell population, however, we found a consistent proportion of these cells in one case which had a transient erythremic picture during the course of the illness A small number of megakaryoblasts may not be uncommon in mixed forms of acute leukemia (myelomonocyt-

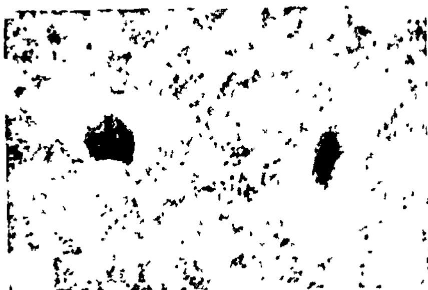


*Fig. 4.* Combined preparation for AS-D Ch-AE + α-NBE + FAS-cyt. The lethal test for lysozyme activity. A large area of lysis is shown around an α-NAE positive cell (mature monocyte, left). A pale decolorat on area is present around a cell (right) with the features of a promonocyte (positive reaction for both the AS-D Ch-AE and the α-NAE) × 1200

ic) or in a terminal phase of the illness, when the blood picture is often recalling the features of a panmyelopathy. In this respect SAARNI and LINMAN [21] have already emphasized that the myelomonocytic forms of leukemia may represent a global involvement of the bone marrow.

The destaining of the preparations provides additional information on the nature of the cells and allows a comparison with the panoptically stained preparates. A misleading interpretation of the Giemsa features is particularly prominent after chemotherapy which alters significantly the morphology of the cells. The characterization of the cells by consecutive cytochemical stainings and the observation of the Giemsa features of the same elements after destaining appear highly valuable for diagnostic purposes and corroborate the opportunity of a double sequence in the cytological identification.

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# Malignant Lymphoma with Plasmacytoid Differentiation and Polyclonal Gammopathy

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*Key Words* Immunoglobulins Lymphoplasmacytoid proliferation Malignant lymphoma Plasma cells Polyclonal gammopathy

*Abstract* Two patients are presented with lymphadenopathy hepatosplenomegaly and infiltration of the bone marrow with plasmocytoid cells in whom an important heterogeneous hyperimmunoglobulinemia was detected (3 18 and 5 85 g/100 ml) Autopsy showed widespread poorly differentiated lymphocytic lymphoma with plasmocytoid differentiation in the first case and a well differentiated lymphocytic lymphoma with plasmocytoid differentiation involving lymph nodes marrow spleen liver and kidney in the second case High concentrations of IgM and IgG were detected in lymph node and spleen homogenates from this case After splenectomy and cyclophosphamide the immunoglobulins decreased It is suggested that both cases had a malignant lymphoma with plasmocytoid differentiation and polyclonal gammopathy

A monoclonal paraprotein may be often seen in lymphoproliferative or plasmocytic disorders [7, 8] In nonneoplasic diseases such as hepatic cirrhosis or chronic infections a polyclonal increase of immunoglobulins is usual, but this is not a common feature of malignant lymphoma In 2 patients we recently observed a polyclonal gammopathy together with a widespread malignant proliferation with plasmocytoid differentiation

## *Case Reports*

*Case 1* M G G a 71 year old male who 2 months before entry complained of anorexia malaise and weight loss 40 days later a macular purpuric rash in the lower extremities and fever appeared Multiple lymph node enlargements were felt In

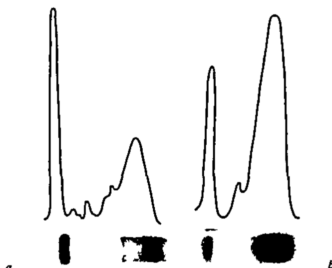


*Fig 1* Serum (a) and urine (b) electrophoresis in case 1, showing a polyclonal increase of immunoglobulins confirmed by immunoelectrophoresis

the abdomen liver and spleen were felt 6 cm below the costal margin Chest X rays showed left hiliary lymph nodes The patient died 10 days later

*Laboratory data* Hematocrit 35%, hemoglobin 11.9 g/100 ml white cell count 16 800 with 11% band forms 64% neutrophils, 21% lymphocytes and 4% monocytes sedimentation rate 120 mm/h Coombs test negative, total proteins 7.1 g/100 ml with 37.79% albumin 1.57% $\alpha_1$ 4.72% $\alpha_2$ 11.02% $\beta$ and 44.8% heterogeneous γ-globulins Immunoelectrophoresis showed a polyclonal increase of all three major immunoglobulins Free heavy chains were not detectable by monospecific antiserums and no M component could be detected in serum or urine In the urine total proteins were 990 mg/24 h, with 17.58% albumin 0.5% $\alpha_1$ 3.51% $\alpha_2$ 5.02% $\beta$ and 73.3% γ globulins (fig 1b) Bone marrow aspiration showed 72% of plasmocytic cells Skull and spine X rays were normal

*Necropsy* There were multiple peripheral lymph node enlargements in thorax and abdomen Their general structure was not preserved there were multiple nodular areas formed by plasmocytoid and some lymphoid cells The capsula was invaded and the most peripheral sinus preserved but the central sinus had generally disappeared The cells were pleomorphic some very big irregular and with prominent nucleolus, while others were small with dense nuclei and scanty protoplasm There were also some plasma cells of normal aspect The spleen was 400 g and invaded by the same cellular elements (fig 2a) There was bilateral pleural and pericardial effusion The lungs showed peribronchial and perivascular infiltrations of lymphoid and plasmocytoid cells and of some reticular cells In the abdomen there was peritoneal effusion The liver weighed 1,500 g and showed some plasmocytoid infiltrations of


a
b
c

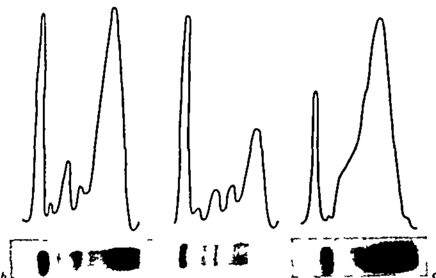


*Fig 3* Serum electrophoresis in case 2 before (a) and 2 months after (b) splenectomy and cyclophosphamide therapy urine electrophoresis (c)

the portal tracts In the gut there was a diffuse infiltration of the lamina propria nodular and focal infiltrations in the submucosa serosa and muscular layers. The esophagus was also infiltrated. In the kidneys there was interstitial infiltration with formation of nodular areas In the skin there was a widespread vascular infiltration without necrosis The bone marrow showed large plasmocytoid infiltration (fig 2b) The adrenals, the pericardium and the heart were also infiltrated by the same type of cells The pathological diagnosis was malignant lymphoma lymphocytic type poorly differentiated with plasmocytoid differentiation

*Case 2* J R M., a 56-year-old male who 8 months before entry presented anorexia weight loss and a dull pain over the left hypochondrium Lymph nodes were felt in both axillae The liver was 3 cm and the spleen 13 cm below the costal margin. At lapartomy several lymph node enlargements were seen in the celiac area and retroperitoneal space Splenectomy lymph node liver kidney and bone marrow biopsies were performed After the operation cyclophosphamide 100 mg daily was instituted and the patient improved. 8 months later he is doing well

*Fig 2* *a* Lymph node from case 1 widespread proliferation of pleomorphic cells, mostly lymphocytes. HE 375 *b* Bone marrow from case 1, diffuse infiltration by plasmocytic cells He 600 *c* Spleen from case 2 marked plasmocytoid differentuation at the right side of the picture HE 600

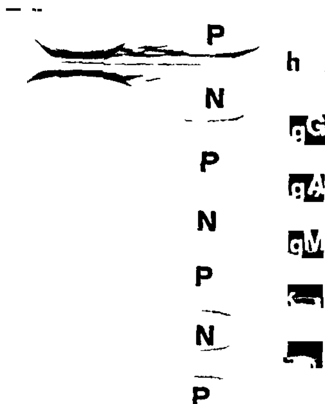



*Fig 4* Immunoelectrophoresis in agarose gel of case 2 showing a polyclonal increase of immunoglobulins specially IgG and IgM h = Antihuman antiserum IgG = anti IgG antiserum IgA = anti IgA antiserum k = anti $\varkappa$ antiserum l = anti $\lambda$ antiserum P = patient s serum N = normal serum

*Laboratory results* Hematocrit 35%, hemoglobin 11 4 g/100 ml white cells 11 000 with 1% eosinophils 9% band forms 67% neutrophils 21% lymphocytes and 2% monocytes Blood urea nitrogen 88 mg/100 ml creatinin 1 6 mg/100 ml uric acid 12 mg/100 ml total proteins 9 2 g/100 ml with 20 23% albumin 2 38% $\alpha_1$ 8 92% $\alpha_2$ 5.35% $\beta$ and 63 69% heterogeneous $\gamma$ globulin IgG was 9 000 mg 100 ml (normal 900–1 800) IgM 900 mg/100 ml (normal 65–150) and IgA 195 mg 100 ml (normal 150–300) Immunoelectrophoresis showed a polyclonal increase of IgG and Igm and no M component could be seen in serum (Fig 4) In the urine proteins were 1 800 mg/24 h with 14 41% albumin 6.51% $\alpha$ 18.5% $\beta$ and 60 46% $\gamma$ globulins (fig 3c) By urine immunoelectrophoresis no free light or heavy chains were detected Bone marrow aspiration showed 22% plasmocytic cells with normal erythroid and myeloid series Immunoelectrophoresis of spleen and lymph node homogenates showed high concentrations of IgG and IgM 2 months after splenectomy the immunoglobulins decreased to half of the previous values total proteins 7 4 g 100 ml with 36 72% albumin 2 16% $\alpha_1$ 8 64% $\alpha_2$ 10 80% $\beta$ and 40 32% $\gamma$-globulins

(fig 3b) 6 months later on continuous cyclophosphamide therapy the serum electrophoresis showed a less marked hypergammaglobulinemia (total proteins 8.3 g/100 ml, 49.3% albumin, 1.2% $\alpha_1$, 7.7% $\alpha_2$, 11.6% $\beta$ and 29.8% $\gamma$ globulins).

*Pathological study.* In the lymph nodes there was a widespread proliferation of lymphoid elements and some plasmocytoid cells. The peripheral sinuses were almost full of these cells and most of the medullary sinuses were also involved. There were heavy iron deposits in the preserved areas. In the spleen the folliculi were big and formed by round monomorphic cells with the chromatin forming clumps. The cytoplasm was scanty. Sinusoids were invaded by the same cells with areas of plasmocytoid differentiation (fig 2c). The liver showed widespread infiltrations of portal tracts by round lymphoid cells. In the kidneys there were areas of lymphoid and plasmocytoid infiltration in the interstitial space. In the bone marrow myeloid and erythroid series were well preserved and there was only a mild plasmocytic reaction. The pathological diagnosis was malignant lymphoma, lymphocytic type well differentiated with plasmocytoid differentiation.

## *Discussion*

Classification of non-Hodgkin lymphomas is under active revision [1, 5, 10, 14, 18]. Among B cell lymphomas are included all diseases characterized by an abnormal production of immunoglobulins, and this is a well known feature of lymphoplasmocytic proliferative disorders, although not always immunoglobulins are secreted [18]. They usually belong to one type of Ig due to the monoclonal character of the neoplasm [7, 8, 18].

Our 2 patients had clinical and pathological data which merit consideration. The finding of an important polyclonal gammopathy is unusual in neoplastic diseases other than hepatoma [2]. In our second case the diminution of the Ig after splenectomy and cyclophosphamide therapy and the high concentrations of IgG and IgM in spleen and lymph node homogenates seem to confirm the relation between the neoplasm and the Ig secretion. The plasmocytic proliferation in the bone marrow could be compatible with multiple myeloma, but the widespread infiltration of lymph nodes, liver, spleen and kidneys without osseous lesions and the type of dysproteinemia do not support this diagnosis.

Recently, FRIZZERA *et al.* [4] described a group of patients of what they call angioimmunoblastic lymphadenopathy with dysproteinemia, and this observation has been confirmed by several other investigators [6, 11, 12, 14, 17]. The histological picture suggests a host versus-graft type of reaction. In our patients the possibility of an immune reaction of humoral

type against the tumor may not be dismissed, although the histological picture is different of that described by FRIZZERA *et al* [4] and [H RAPPAPORT, personal commun ]

KIM *et al* [7], in their revision of lymphoproliferative disorders with monoclonal gammopathies, found 6 cases with a moderate polyclonal increase of IgM and pronounced plasmocytoid features which may well be a less severe form at the same type of clinicopathological picture FLANDRIN *et al* [3] reported 7 patients of what they call plasmocytic sarcomatosis in whom there was also a polyclonal hyperimmunoglobulinemia, although their clinical records were different from those of our patients In Hodgkin's disease a splenic synthetsis of IgG stimulated by tumor-related antigens has been demonstrated [9], but this diagnosis cannot be maintained in our 2 cases Neither of them belong to the double macroglobulinemia-myeloma gammopathy recently reviewed [13] Heavy chain diseases can be excluded because the serum immunoelectrophoresis and urine in case 2 and of the serum with monospecific antiserum, did not show free heavy chains

The widespread proliferative disease in our 2 cases seems to correspond to a malignant neoplastic disorder with polyclonal gammopathy Although specific B cell markers were not determined it is suggested that the tumor may arissey in B cells As recently suggested by SALMON and SELIGMAN [16] this may be a $B_0$ cell type of neoplasm Only reports of further cases of such a type of disease will confirm this

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# Splenunculectomy in Recurrent Thrombocytopenia [1]

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*Key Words* Accessory spleen ITP Splenectomy Splenunculectomy Thrombocytopenia

*Abstract* A 13 year old boy developed thrombocytopenic purpura in 1953 which improved during 4 weeks of cortisone therapy Following 13 years of intermittent symptoms the platelet count was found to be 8 000/µl After splenectomy the patient was asymptomatic for 8 years but had a recurrence of symptoms and thrombocytopenia in 1974 An initial spleen scan with $^{99}$Tcm sulfur colloid was negative, but when repeated with a gamma camera and shielded liver, a splenunculus was demonstrated After splenunculectomy a rapid remission occurred and the patient has been well for 13 months

The search for and removal of an accessory spleen (lienculus lienunculus, splenculus, splenulus or splenunculus) in a patient with recurrent thrombocytopenia following splenectomy seems to be a forgotten mode of treatment for this recalcitrant disease The last reports and or reviews [2, 8 9] were published in the 1950s Current literature emphasizes the use of immunosuppressive agents or corticosteroids [3, 6] This case is presented as a reminder that it is still worthwhile hunting for a splenunculus because its excision may induce a rapid remission

## *Case History*

The patient is a 35 year old male who in July 1953 at age 13 was diagnosed as having acute idiopathic thrombocytopenic purpura (ITP) which responded to steroid

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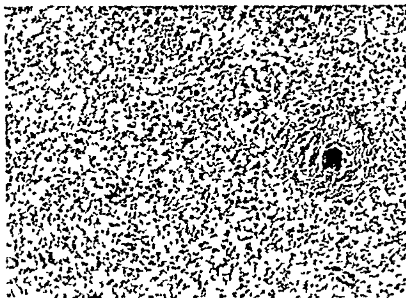


*Fig. 1.* Microscopic section of splenunculus. ×75.

therapy. In the spring of 1966 he was contacted and asked to come to this laboratory as part of a study of individuals who had had ITP [10]. A history of intermittent episodes of spontaneous petechiae, easy bruising, gum bleeding and epistaxis during the past 13 years was obtained. No treatment had been instituted. Physical examination showed ecchymotic areas on both arms and one thigh. Spleen was not palpable. Platelet count was 8,000/µl and bone marrow aspiration and biopsy revealed a normal number of megakaryocytes. LE preparation was negative.

He was treated with prednisone, 100 mg/day. Because he was unable to maintain safe platelet levels when steroids were tapered, a splenectomy was performed in December 1966. The spleen weighed 130 g and histology was normal. An accessory spleen weighing 0.5 g was also excised at surgery. Within days his platelet count rose to greater than a million. Steroids were stopped, and he was followed for 2 years during which time he maintained platelet counts greater than 350,000/µl. He remained asymptomatic for the ensuing 5 years, and during a hospitalization for a herniorrhaphy in 1973, his platelet count was reported as normal. In mid April 1974, more than 7 years post splenectomy, he noted a recurrence of easy bruisability, but did not seek medical advice until late in May when his platelet count was found to be 25,000/µl. Treatment with prednisone resulted in a temporary increase in platelet count, but by early July 1974 his count again fell markedly with tapering and of the medication. In mid July a conventional rectilinear scan of the upper abdomen using 99Tcm sulfur colloid showed no significant concentration of radioactivity outside of the liver. Because the possibility of an accessory spleen was strongly suspected, the

study was repeated 2 weeks later utilizing a gamma-camera Images were obtained of the left upper quadrant with lead placed over the liver, thereby emphasizing the recording of extrahepatic radioactivity With this technique a significant amount of the radiopharmaceutical was demonstrated in the splenic bed, and it was felt to represent an accessory spleen of approximately 20 g On August 6, 1974 a laparotomy was performed and an accessory spleen weighing 12.8 g was found (fig 1) Prolonged search revealed no other macroscopic splenic tissue Following surgery his platelet count rose to a peak of 455 000/μl and was 280 000 13 months after operation The patient was clinically well at the time of his most recent platelet count

## *Discussion*

In the early 1950s several reports appeared in the literature discussing the importance of accessory spleens in recurrent ITP Since that time it appears that interest in pursuing a splenunculus in this setting has waned Considering the marked improvement in diagnostic procedures in the last 20 years, especially in the area of nuclear medicine, the tendency to move directly to drug therapy without exhaustive search for residual splenic tissue may well be doing a disservice to a small percentage of patients with recurrent ITP

The presence of accessory spleens in necropsy series varies from 10 [4] to 31% [1], however, their occurrence following splenectomy is not as well documented It has been shown that more than 10% of those patients with one accessory spleen have been found to have additional splenunculi [1, 4, 5], the implication being that a patient who has an accessory spleen at initial surgery has at least a 10% chance of having additional aberrant splenic tissue Howell-Jolly bodies were not seen in this patient's peripheral blood smears, and this was taken as an additional indicator of the presence of functioning splenic tissue

These two factors, i e the previous finding of a splenunculus and the lack of Howell-Jolly bodies, led to the heightened index of suspicion that prompted continued searching following the initial negative scan and the subsequent radiologic demonstration of the existence of an accessory spleen The prior intention in this patient had been to proceed with a heat-treated, $^{51}$Cr-labeled red blood cell study [7] if the repeat $^{99}$Tc$^{m}$ spleen scan using a long exposure gamma camera and liver shielding had been negative This procedure is based upon the observation that the splenic uptake of red blood cells is said to be significantly greater than that of the liver If all studies had been negative, in all likelihood drug

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therapy would have been continued Random exploratory laparotomies in the absence of evidence for splenic activity are not justifiable

A vigorous search for an accessory spleen in all patients with recurrent thrombocytopenia following splenectomy induced remission, especially those in whom the index of suspicion is raised, may avoid the potential hazard and expense of prolonged chemotherapy

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# Multiple Myeloma with Terminal Erythroleukemia

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*Key Words* Erythroleukemia · Melphalan · Myeloma · Preleukemia

*Abstract* A patient with multiple myeloma treated by melphalan terminated in erythroleukemia. The evolution of the leukemia was preceded by a protracted preleukemic phase, as evidenced by progressive pancytopenia. The possible implications of an early diagnosis of preleukemia in myeloma are discussed.

A number of case reports of acute myeloid or myelomonocytic leukemia as the terminal event in multiple myeloma have been published [6]. Recently, sideroblastic anemia was described as a forerunner of this leukemia [3]. The following report presents the evolution of erythroleukemia in a patient with multiple myeloma.

## *Case Report*

This 72 year old Jewish female was diagnozed in 1965 as suffering from multiple myeloma. Except for cholelithiasis and recurrent urinary tract infections, the past history was uneventful. On admission she had generalized osteoporosis, an asymptomatic compression fracture of $L_1$ vertebra and osteolytic lesions of the skull. Anemia of 9 g% hemoglobin and hyperglobulinemia with 4.5 g% globulins were present. The rest of the blood count, blood chemistry and urinalysis were normal. Serum electrophoresis demonstrated a *monoclonal gammopathy*. This was identified as IgA/λ. The bone marrow aspirate was hypercellular and contained 50% mature plasma cells, normal myeloid series and normoblastic erythropoiesis. Since this relatively old lady was asymptomatic, no specific treatment was instituted.

In November 1969, following a nailing for a hip fracture and gram negative septicemia, an episode of hypercalcemic coma developed. A prompt response was

*Table I* The patient's course serial blood counts, immunoglobulin levels and melphalan therapy

| Period | Peripheral blood | | | | | Immunoglobulins, mg% | | |
|---|---|---|---|---|---|---|---|---|
| | hemoglobin g% | leucocytes/mm³ | platelets/mm³ | blasts % | normoblasts per 100WBC | IgA | IgG | IgM |
| 1965 1969 | 10 6 – 11 0 | 4 000 – 5,700 | 180 000 – 200 000 | 0 | 0 | | | |
| Melphalan | | | | | | | | |
| Apr 70 Jan 73[1] | 9 7 – 13 1 | 3,500 – 6 300 | 110 000 – 230 000 | 0 | 0 | 4,350 | 180 | 47 |
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[1] Cyclic melphalan therapy without interruption

[2] Melphalan repeatedly discontinued every 2 3 months

[3] Melphalan completely withdrawn

achieved by buffer phosphate infusion and prednisone At that time immunoglobulin levels were 4,350 mg% IgA 180 mg% IgG and 47 mg% IgM Subsequently cyclic therapy with melphalan (2–4 mg daily for 1 week with 2 weeks off therapy) and intermittent prednisone was instituted On this regimen the patient remained asymptomatic during the following 4 years. The hemoglobin rose to 13 g%, and the IgA of the monoclonal type decreased gradually to 300 mg% with a concomitant rise in IgG to 680 mg% and IgM to 95 mg% (table I)

Starting from January 1973 melphalan had to be repeatedly discontinued because of leucopenia Since October the hemoglobin level started to drop progressively and in April 1974 a persistent pancytopenia developed Numerous normoblasts (from 25 to 50 100 WBC) poikilocytes, schistocytes, basophilic stippling and occasional myeloblasts were visible on the blood smear Most of the normoblasts were ringed sideroblasts and 30% were strongly PAS positive The bone marrow aspirate was hypercellular with only 5% mature plasma cells. The erythroid series was markedly hyperplastic with a shift to the left Numerous double nucleated normoblasts with prominent siderotic granules and ringed sideroblasts were present In the myeloid series except for a shift to the left with up to 1% blasts, no abnormality was observed The serum iron was 166 mg% and IBC 133 mg% and the LAP score 244 100 WBC

In view of the pancytopenia melphalan therapy was discontinued and the patient remained asymptomatic on 30 mg prednisone daily however the blood count dropped further The serial counts are presented in table I Cytogenetic examination of the peripheral blood, performed in December 1974 demonstrated 2 aberrant metaphases out of 10 scorable cells 46,xx G+F and 47 xx+C respectively In the repeated bone marrow the erythropoiesis was even more pathological with triple nu

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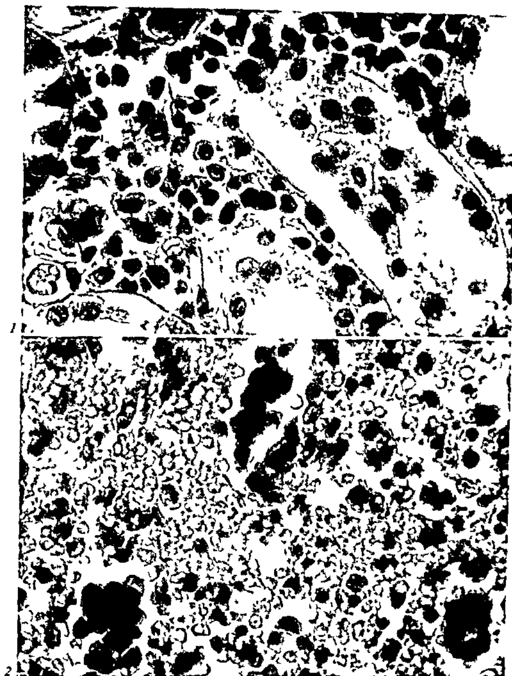


*Fig 1* Erythroleukemic infiltrate in kidney HE × 630
*Fig 2* Clusters of abnormal erythropoietic cells in spleen HE × 1,000

cleated normoblasts and megaloblasts. Also the megakaryocytes showed asynchronic maturation

The patient died of a massive bronchopneumonia by the end of December 1974 At autopsy erythroleukemic infiltrates were found in the bone marrow spleen, liver kidney and occasional lymph nodes (fig 1, 2)

## *Discussion*

Erythroleukemia, a rare form of acute leukemia, is more frequent following potentially leukemic conditions Thus, 3 of 6 patients with leukemia post polycythemia vera [5] 3 of 7 patients with leukemia following macroglobulinemia [1, 6] and 4 of 74 patients with leukemia post Hodgkin's disease [7] terminated in erythroleukemia However, in a recent review of leukemia post myeloma only in 1, of all 46 cases culled from the literature, erythroleukemia evolved [6]

This patient demonstrated a number of additional aspects during a period of 4 years she had a documented multiple myeloma, including bone destruction However, since the policy of this department is not to treat subjectively asymptomatic patients she was followed for 4 years without any specific therapy Due to a fracture followed by septicemia with hypercalcemic coma the patient was put on melphalan, using a relatively conservative schedule She responded remarkably, the pathological protein dropped significantly, the normal immunoglobulins and the hemoglobin rose and the patient resumed normal life Therefore, at least in this case, the delay in instituting therapy did not change her responsiveness to melphalan This appears to be quite a consistent observation in our material [4] The last stage of the disease was characterized by difficulties in pursuing melphalan therapy because of repeated pancytopenia Finally, erythroleukemia evolved Retrospectively, it is clear that the cytopenia was related to the preleukemic phase, and was not due to myelosuppression by the drug

A preleukemic phase, consisting of pancytopenia and sideroblastic marrow, has recently been described preceding myelomonocytic leukemia [8] and the acute leukemia post multiple myeloma [3] as well as post macroglobulinemia [1] polycythemia vera [5] and Hodgkin's disease [9] Therefore preleukemia should be borne in mind when considering the various causes of cytopenias in patients who receive myelosuppressive therapy for potentially leukemic conditions In view of the strong implication of melphalan in the evolution of acute leukemia in multiple myeloma

[2], the demonstration of preleukemia should call for a complete withdrawal of this drug

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# Index rerum ad Vol. 55

Bearbeitet von G. Boehm, Basel

(B) = Book Reviews – Buchbesprechungen – Livres nouveaux

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*Table V.* Patient's lymphocyte stimulation in culture using penicillin, ampicillin and cephalothin as antigen performed on 2 different days (mean of 2 tests)

| | 16.8.70 cpm | 18.9.70 cpm |
|---|---|---|
| Lymphocytes alone | 513 | 472 |
| Lymphocytes + penicillin G | 971 | 728 |
| Lymphocytes + ampicillin | 1,119 | 884 |
| Lymphocytes + cephalothin | 1,096 | 1,216 |
| Lymphocytes + PPD (10 µ) | 1,582 | 1,390 |
| Lymphocytes + phytohemagglutinin | 63 620 | 54 620 |

way. Moreover (table IV), no clear-cut difference could be seen between the fractions from the patient's serum and those from normal serum. The serum proteins most readily absorbed by this apparently nonimmunologic mechanism were IgG, IgA, transferrin and β-lipoprotein.

*Evidence for sensitization to cephalothin.* Skin tests were performed in the patient, 9 days after therapy with cephalothin had been interrupted. A positive skin test was observed after intradermal injection of penicilloyl-polylysine, 250 mmol/ml), the last test was stronger than the others. Lymphocyte culture tests were also performed on two different days, using the same three drugs as antigens (table V). All three tests induced moderately positive lymphocyte stimulation.

## *Discussion*

The reported incidence of positive Coombs test in patients receiving cephalothin has varied between 3.4 and 75% [4, 7, 9, 11]. These reactions were often weak and occurred from one to a few days after starting treatment. They were not associated with hemolysis. In some studies, the dosage and duration of therapy were found to influence the incidence of positive reactions. A large percentage of patients with positive Coombs tests had renal disease with high blood levels of cephalothin secondary to reduced renal excretion. As suggested by GRALNICK *et al.* [4], hypoalbuminemia may also favor the occurrence of positive Coombs tests, the albumin fraction being responsible for over 95% of the serum binding of cephalothin [6].

Previously proposed mechanisms regarding the pathogenesis of posi-

tive Coombs test without hemolysis, occurring in patients receiving cephalothin include (1) a nonimmunologic binding of serum proteins to red cells [4, 7, 11] and (2) an immune reaction involving an antibody cross reacting with cephalothin or penicillin-coated red cells [4, 8, 11] As indicated by SPATH *et al* [11], both mechanisms seem to occur in parallel in these patients Nonimmunological absorption of proteins, especially albumin, to cephalothin-coated red cells could be demonstrated *in vitro*, multiple absorptions of a serum with cephalothin sensitized erythrocytes revealed that these cells absorb IgG and other proteins, including complement (C3) and albumin [11] Our own data confirm this nonimmunologic binding of proteins and revealed that IgA, transferrin and $\beta$ lipoprotein may also be absorbed from normal sera by cephalothin-coated erythrocytes (table III) In addition SPATH *et al* [11] have demonstrated that an immune mechanism may also cause positive direct antiglobulin tests *in vivo* during cephalothin therapy Eluates from the patient s erythrocytes react with cephalotin and penicillin sensitized erythrocytes This was not a nonspecific absorption of protein because eluates from red cells sensitized with an anti Rh IgG isoantibody would not bind IgG to cephalothin-coated red cells lacking the appropriate antigen In addition, SPATH *et al* [11] showed that the sera of 4 patients having a positive antiglobulin test all contained high titers of IgG cephalothin antibodies, whereas in the other treated patients, IgM antibodies were found in 88% and IgG antibodies in only 25% of cases

Despite the frequent occurrence of a positive direct Coombs test in patients receiving cephalothin only 2 cases of hemolytic anemia associated with cephalothin have been described so far [5] As in our case, erythrocytes from these patients were strongly positive in the direct Coombs test with broad spectrum anti human globulin reagents and with a specific anti IgG antiserum Our patient s erythrocytes were also strongly agglutinated by an anti-complement (C3) reagent Weak reactions were also obtained with anti IgM anti IgA and anti transferrin reagents, suggesting the simultaneous occurrence of nonimmunological binding of serum proteins In our case as in the others a specific IgG cephalothin antibody was demonstrated in eluates from patients erythrocytes However, as mentioned above this specific immune reaction was also observed in patients with positive direct Coombs test without hemolysis and therefore cannot be used as a definitive proof that cephalothin caused the hemolytic anemia Presumably as suggested by WORLLEDGE [12] the quality and quantity of the antibody are also important.

Several feature suggest that our patient was actually sensitized to cephalothin. Skin tests performed after therapy had been interrupted, revealed sensitization to cephalothin, but also to penicillin and ampicillin, two drugs the patient had received previously. In addition *lymphocyte* stimulation was observed in culture tests *in vitro* using the same three drugs as antigens. The possible role of cephalothin in hemolytic anemia is further suggested by the fact that evidence of hemolysis was noted within 2 weeks after therapy had been initiated, and disappeared quickly when the drug was stopped. The direct anti-globulin test gradually became weaker until it was found negative, 60–70 days later, when the blood level of cephalothin was very low in the blood and the anemia much improved. This patient illustrates the complexity of interpreting immunological tests involving drug interacting with red blood cell membranes.

*Acknowledgements.* We thank MISS RACHEL BALLARD and MR. P. MEYLAN for valuable technical assistance.

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# Simultaneous Occurrence of Myeloma and Hodgkin's Disease

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*Key Words* Hodgkin's disease  Multiple malignancies  Myeloma  Paraproteins

*Abstract* A patient in whom lymphocyte-depleted Hodgkin's disease and multiple myeloma were diagnosed simultaneously is reported There was no prior history of irradiation or chemotherapy, the significance of which is discussed in relation to previously documented patients with Hodgkin's disease and myeloma

The coexistence of Hodgkin's disease and multiple myeloma has only been reported in three patients [4, 7] In each of these instances, myeloma was diagnosed after either irradiation or chemotherapy had been given for the Hodgkin's disease, raising the possibility that myeloma may have been induced, or precipitated, by these therapeutic interventions The purpose of this report is to present a fourth patient in whom both Hodgkin's disease and multiple myeloma were diagnosed simultaneously, in the absence of previous irradiation or chemotherapy

## *Case Report*

A 51-year-old fisherman began experiencing recurrent fever up to 40°C in December 1973 associated with intermittent rigors and night sweats A 9 kg weight loss ensued over the following 7 months The fever had a classical Pel Ebstein periodicity

... hepatosplenomegaly, ... 00 with ... platelet

count 250,000/μl, sedimentation rate 107 mm/h, total protein 7 8 g/100 ml, and albumin 3 7 g/100 ml A serum protein electrophoresis showed a monoclonal spike in the gamma

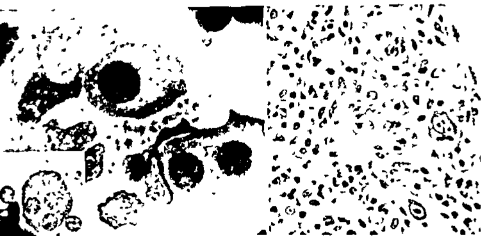


*Fig 1* Bone marrow smear stained with Wright-Giemsa, showing abnormal plasma cells with some double nucleated forms containing nucleoli ×930 *Inset* Plasma cell with four nuclei ×705

*Fig 2* Section of bone marrow biopsy, stained with hematoxylin-eosin, showing pleomorphic cellular features with abnormal 'reticulum cells', some containing large nucleoli Note the double nucleated reticulum cell with prominent nucleoli, resembling a Reed-Sternberg cell ×210

region approximating 1 7 g%. Immunoelectrophoresis revealed a monoclonal IgG-kappa paraprotein with marked attenuation of normal immunoglobulins Free kappa light chains were detected in the urine in low concentration A quantitative T- and B-lymphocyte study revealed absolute lymphocytopenia (252 µl) with a relative increase in the numbers of IgG kappa, B-lymphocytes. BUN, calcium, roentgenographic bone density, chest X-ray and bone survey were all normal Liver function tests were normal, except for an increase in alkaline phosphatase (isoenzyme of liver origin) and bilirubin

A bone marrow aspirate from the iliac crest demonstrated a mild infiltrate of primitive plasma cells, many of which were multinucleated with prominent nucleoli (fig 1) A needle biopsy of the bone marrow revealed an area of pleomorphic cellular infiltration, mild fibrosis and numerous primitive reticulum cells, some recognizable as Reed Sternberg cells (fig 2) A lymphangiogram demonstrated enlarged iliac and paraaortic nodes Laparotomy revealed Hodgkin s disease of the lymphocyte-depleted type involving the liver, spleen (420 g) and retroperitoneal lymph nodes.

The patient was treated with six cycles of nitrogen mustard, vincristine, procarbazine and prednisone (MOPP) at monthly intervals, with immediate resolution of fever, rapid weight gain and return of well-being After 6 months of therapy he felt normal and the liver function tests had returned to normal The paraprotein concentration remained unchanged at 1 8 g 100 ml and the sedimentation rate was 60 mm h A repeat marrow examination was normal apart from persistence of the plasma cell infiltrate Free light

chains were now absent from the urine He enjoyed a complete remission from Hodgkin's disease lasting 11 months during which time he received four cycles of MOPP (excluding prednisone), at approximately 2 month intervals as maintenance therapy

In December 1974 he again experienced intermittent fever up to 104°F, rigors, sweats and weight loss Physical examination was normal Hemoglobin 10 2 g/100 ml, white cell count 6,500/μl, platelet count 151,000/μl, sedimentation rate 122 mm/h, total protein 7 4 g/100 ml and albumin 3 8 g/100 ml A bone marrow needle biopsy revealed hypoplastic marrow with mild plasmacytosis and Hodgkin's involvement Treatment with MOPP resulted in only transient fever control and caused significant pancytopenia (platelet count 34,000/μl, white cell count 2500/μl) precluding further therapy Bleomycin, 30 U i v twice a week, and vincristine, 1 5 mg i v weekly, was commenced, together with prednisone 30 mg daily, with rapid relief of symptoms He has remained in remission to the time of writing (4 months since beginning Bleomycin) His latest blood count in April 1975, showed hemoglobin 11 8 g/100 ml, white cell count 9,200/μl and platelet count 360 000/μl The paraprotein level was approximately 1 1 g/100 ml and quantitative immunoglobulin analysis showed IgG 1,770 mg%, IgA 37 mg%, IgM 25 mg%. The patient is being maintained on Bleomycin 10 U and vincristine 1 0 mg weekly i v and 20 mg prednisone by mouth on alternate days

## *Discussion*

It is now well established that there is an increased risk of second neoplasms associated with Hodgkin's disease with an overall incidence of 1 6 to 2 2% [3, 9, 10] In a study of 425 patients with Hodgkin's disease, ARSENEAU *et al* [2] observed a 3 5-fold increase in the risk of developing a second malignancy in all patients, with the greatest risk (29-fold) in a group of 35 patients who had received both intensive radiotherapy and intensive chemotherapy The spectrum of second malignancies in Hodgkin's patients is broad [4, 10, 13], acute leukemia having been particularly emphasized [5, 11, 12, 14] The increased incidence of second neoplasms in Hodgkin's patients has been attributed to the cellular immune deficiency associated with the disease as well as the immunosuppressive effects of radiotherapy and chemotherapy

The occurrence of paraproteinemia in Hodgkin's disease is very rare, having been identified in one study in only 1 of 218 patients [1] Overt myeloma has been documented in association with Hodgkin's disease in three patients previously [4, 7] The patient presented in this report is the first in whom myeloma and Hodgkin's disease were diagnosed simultaneously prior to irradiation therapy or chemotherapy, thus excluding these immunosuppressive modalities from the casual relationship between the two neoplasms The probability that previous immunosuppressive therapy

is not an important factor in the association between Hodgkin's disease and myeloma is further supported by the first case reported by GREENBERG *et al* [7] in whom the interval between irradiation therapy and the diagnosis of florid myeloma could not have been more than 3 months. The diagnosis of myeloma in our patient seemed clearly established by the triad of an IgG-kappa paraprotein, kappa light chains in the urine and myeloma cells in the marrow aspirate. The simultaneous occurrence of Hodgkin's disease and myeloma may represent a chance association of two uncommon malignancies. Alternatively, cellular immune deficiency as in chronic lymphocytic leukemia and various congenital immunodeficiency diseases [6, 8] may be important in predisposing Hodgkin's patients to an increased risk of developing other neoplasms, including myeloma. There are no reports in the literature of Hodgkin's disease developing at some time after the diagnosis of myeloma, suggesting that it is more likely that Hodgkin's disease predisposes to myeloma than the reverse.

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# Plasmocytoma with IgM Paraproteinemia. A Case Report

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*Key Words* Macroglobulinemia Paraproteins Plasmocytoma

*Abstract* A case of typical diffuse plasmocytoma with IgM paraproteinemia is described The patient presented with systemic and monomorphic proliferation of atypical and immature plasma cells, osteolytic lesions, retinal venous thrombosis and hemorrhagic diathesis. The therapy with steroids and alkeran produced clinical and hematological remission for $1^1/_2$ year with a decrease of macroglobulins in the serum Cell and paraprotein type remained unchanged during the course of the disease This case, and similar ones already reported in the literature suggest that neoplastic plasma cell proliferation is accompanied by synthesis of paraproteins including the IgM type However, this is not characteristic for Waldenstrom's macroglobulinemia The author concludes that differential diagnosis in these cases can only be made by cytologic examination

Proliferating neoplastic plasma cells synthesize 'monoclonal' immunoglobulins of different types (G, A, M, D, E), and/or exclusively light chains IgM plasmocytoma is very rare, thus differential diagnostic problems with Waldenstrom's macroglobulinemia might arise Monoclonal macroglobulinemia has been found in patients showing atypical cytological findings resembling those of Waldenström's cases, and in some patients with typical plasmocytoma These cases have been differently interpreted and classified In this paper, a typical case of diffuse plasmocytoma with IgM paraproteinemia, hemorrhagic diathesis and osteolytic lesions is described

## *Case Report*

Patient M R, a 53 year-old man, admitted to the Policlinico Gemelli in October 1969 with a 4 month history of recurrent bronchitis, pallor, progressive reduction of

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Proliferating neoplastic plasma cells synthesize 'monoclonal' immunoglobulins of different types (G, A, M, D, E) and/or exclusively light chains. IgM plasmocytoma is very rare, thus differential diagnostic problems with Waldenström's macroglobulinemia might arise. Monoclonal macroglobulinemia has been found in patients showing atypical cytological findings resembling those of Waldenström's cases, and in some patients with typical plasmocytoma. These cases have been differently interpreted and classified. In this paper, a typical case of diffuse plasmocytoma with IgM paraproteinemia, hemorrhagic diathesis and osteolytic lesions is described.

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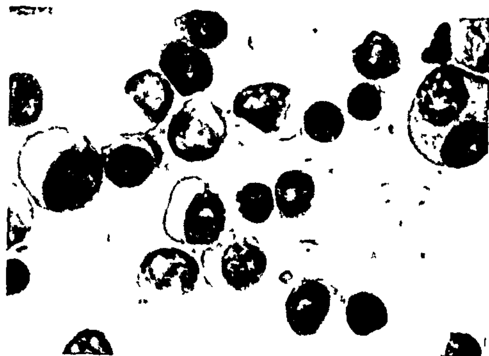


*Fig. 1* Sternal marrow proplasmocytes and plasmoblasts May Grunwald Giemsa × 800

vision and repeated epistaxis On physical examination he presented great pallor and apical systolic murmur Examination of the fundus revealed bilateral thrombosis of central retinal veins

Erythrocytes 1 860 000 $/\mu l$ Hb 4 2 g% white blood cells 5 800 $/\mu l$ with 40% neutrophils 53% lymphocytes 5% monocytes 2% myelocytes Erythrocytes presented anisocytosis and rouleaux formation Platelets 80 000 $/\mu l$ Sternal puncture showed marked cellularity 70% of which composed mostly by proplasmocytes and by some plasmablasts with light basophilia generally eccentric nuclei and prominent nucleoli (fig 1) The normal myeloid cells were altogether scarce Sedimentation rate 90 mm/h Total serum proteins, 11 6% Serum electrophoresis on cellulose acetate showed albumin 27% $\alpha_1$ globulin 3% $\alpha_2$ globulin 6% $\beta$ globulin 7% $\gamma$ globulin 57% The $\gamma$-peak is of the monoclonal type The serum immunoelectrophoresis with polyvalent and specific immunoglobulin antisera IgG IgA IgM IgD IgE showed a very thin but normal IgG and IgA precipitation line while the IgM line was deformed and very thick (fig 2) The macroglobulins separated on Sephadex G 200 were formed by light chains of the $\lambda$ type Immunoglobulin levels with radial immunodiffusion IgG 160 mg% IgA 20 mg% IgM 4 200 mg%

Serum ultracentrifugation showed a large fraction (40 5%) of 16 7 S The serum kept at 4 °C showed a modest amount of cryoglobulin precipitate The SIA test was positive There was no proteinuria Tourniquet test was negative bleeding time

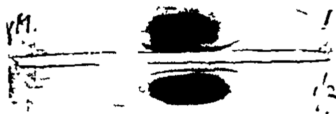


*Fig 2* Immunoelectrophoretic patterns of serum proteins with specific IgM rabbit antiserum

6 min, coagulation time 3 min 30 sec Quick time 19 sec prothrombin activity 35%, fibrinogen 70 mg%. Thromboelastogram showed R 24 min and MA 20 mm Blood urea nitrogen 18 mg%, creatinin clearance 126 ml min blood calcium 9.6 mg%, total cholesterol 75 mg%. Complete and incomplete erythrocyte antibodies were absent Rheuma and Waler Rose tests were negative X ray examination of chest bones, vertebral column and skull showed no alterations.

The patient was treated with blood transfusions, prednisone (100 mg day) and alkeran (8 mg day) Clinical conditions progressively ameliorated and after several therapy cycles the situation was as follows (October 1970) erythrocytes, 4 700 000 μl Hb 14.3 g%, white blood cells 5 900 μl platelets 340 000 μl Total serum proteins, 6.8 g% with 26% of γ-globulins Immunoglobulin levels IgG 400 mg%, IgA 13 mg%, IgM 440%. The sternal marrow showed repopulation of normal myeloid cells with 1% plasma cells. The coagulation tests were normal

In June 1971 the patient commenced to suffer from progressive chest pains, fever pallor and epistaxis X ray examination showed marked osteoporosis of the vertebral column and ribs, conical crush of thoracic vertebrae VI VII and XI and microalveolar skull structure The patient was again admitted and treated as previously but his clinical conditions worsened (anemia, bone lesions, hemorrhagic diathesis with epistaxis, hematemesis and melena) In December 1971 the following data were available erythrocytes, 2 600 000 μl Hb 7.2 g%, white blood cells 4,500 μl platelets 80 000 μl Total serum proteins, 12.3 g%. Serum electrophoresis showed 61% γ-globulins with monoclonal peaks Immunoglobulin levels IgG 140 mg%, IgA 30 mg%, IgM 1800 mg%. Immunoelectrophoresis showed a deformed and very thick IgM line Quick time 15 sec prothrombin activity 62%, fibrinogen 86 mg%, coagulation time 12 min bleeding time 5 min Blood calcium 13 mg%. There was no proteinuria The sternal marrow was composed almost exclusively by plasma cells

The patient died in February 1972 Autopsy revealed pulmonary edema with bilateral pneumonic foci, turbid myocardium, gastric and intestinal hemorrhages, splenomegaly with hyperplasia of the red pulp and indistinct Malpighian bodies, plasma cell nodules in the vertebrae and ribs Histology showed plasma cell infiltrations in vertebrae, ribs, liver, spleen and kidneys (fig 3)

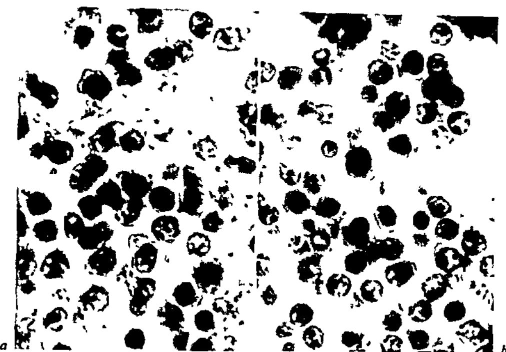


*Fig 3* Histologic patterns monomorphic infiltration of plasma cells in the spleen (a) and the dorsal vertebrae (b) HE. × 400

## *Discussion*

The described case is a diffuse plasmocytoma characterized by (1) *monomorphic proliferation of atypical immature plasma cells in the bone* marrow, liver, spleen and kidneys, (2) osteolytic lesions in the skull, vertebrae and ribs, and (3) hemorrhagic diathesis with prolonged bleeding and coagulation times, reduced prothrombin activity and hypofibrinogenemia An early symptom was reduction of vision due to bilateral thrombosis of central retinal veins Paraproteinemia was of IgM type, λ-light-chains, 16 7 S The cellular aspects and paraprotein pattern remained constant during the course of the disease, although they were quantitatively reduced by steroid and alkeran therapy which induced a $1^1/_2$-year period of clinical and hematological remission

*The case presented the typical cellular aspect* of plasmocytoma and the paraprotein type of Waldenstrom's macroglobulinemia Difficulty arised for differential diagnosis and nosologic classification since some cases considered as plasmocytoma or plasma cell leukemia with macroglobulin-

emia, presented histologic aspects of the lympho-plasmocytic or lympho-reticulo-plasmocytic type, intermediate between plasmocytoma and Waldenstrom's macroglobulinemia [4, 8, 9, 14]. These cases, however, because of the cellular polymorphism they present are closer to Waldenstrom's disease than to plasmocytoma. It should also be considered that in these cases, it is difficult to establish if the synthesis of 'monoclonal' immunoglobulins is due to neoplastic immunocompetent cells or to immunologically reactive phenomena. Moreover, recent studies have shown the frequency of osteolytic lesions in Waldenström's macroglobulinemia, similar to those found in plasmocytoma [15].

On the other hand, the existence of typical diffuse plasmocytoma with IgM paraproteinemia has been demonstrated [1, 2, 11, 13, 16]. IgG and IgM double paraproteinemia has been recognized in 1 case of diffuse plasmocytoma [10], in liver solitary plasmocytoma [12] and in plasma cell leukemia [6]. IgM paraproteinemia and IgG heavy chains have been found in 1 case of plasma cell leukemia [5]. All these cases, plus the experimental evidence in BALB mice [3, 7], show that neoplastic plasma cells can synthesize IgM as well as the other types of immunoglobulins. Therefore, the presence of a certain immunoglobulin type alone cannot differentiate the various paraproteinemic diseases. The terms 'Waldenström's macroglobulinemia type' or 'myeloma type' paraprotein are thus unsuitable to designate the 'monoclonal' synthesis of IgM or of the other classes of immunoglobulins. Since the presence of IgM paraproteinemia is by no means pathognomonic of Waldenström's disease and since osteolytic lesions are not exclusive of plasmocytoma, differential diagnosis and classification of the different forms of paraproteinemic hemoblastoses can be best made by cytologic examination.

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# Autologous Marrow Reconstitutions in Severe Aplastic Anaemia after ALG Pretreatment and HL-A Semi-Incompatible Bone Marrow Cell Transfusion[1]

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*Key Words* Antilymphocyte globulin Aplastic anaemia Bone marrow transplantation Marrow reconstitution (autologous)

*Abstract* Three consecutive patients considered to have end-stage acquired aplastic anaemia were given 100–160 mg/kg antilymphocyte globulin (ALG) i v followed by an infusion of 2–3 8 × 10⁸ nucleated marrow cells/kg i v from HL-A one haplotype identical, MLC-positive family donors. All patients showed autologous marrow reconstitutions lasting now 2–3½ years. No clear-cut evidence of marrow engraftment could be established and no graft versus-host disease was seen. It is assumed that these patients had some normal pluripotent haemopoetic stem cells which proved to be able of endoreduplication and of going into cycle after ALG conditioning and allogeneic marrow transfusion

Acquired aplastic anaemia (AA) is a poorly understood entity with a bad prognosis [1–6] Several aetiological factors may be responsible for the disorder, in particular failure of the haemopoetic stem cell, disturbance of the bone marrow matrix, and potentially a disturbance in the humoral factors which regulate haemopoesis [6]

Treatment with anabolic steroids and glucocorticosteroids as well as improved supportive care with antibiotics, platelet and granulocyte transfusions have not markedly improved the prognosis of this disorder [2, 4, 5] Marrow grafting after conditioning with high dose cyclophosphamide

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has opened new possibilities for treatment of patients with AA. However, such treatment has so far only been successful if HL-A-identical, mixed lymphocyte culture (MLC)-negative siblings have been available as marrow donors [7]. In HL-A-mismatched situations, fatal graft versus host disease (GvH) has always ensued. Conditioning with heterologous anti-lymphocyte globulin (ALG) has resulted in split chimaerism in some experimental systems [8, 9] and in several clinical patients [10–12].

However, marrow engarftment was only transient but in a few instances followed by autologous marrow regeneration. GvH was absent despite histoincompatibility in clinical and experimental grafts after this type of conditioning. We are reporting three consecutive patients with long-lasting marrow aplasia who were given marrow cell transfusions from HL-A semi-incompatible, MLC-positive family donors after conditioning with i.v. ALG.

## *Case Reports*

*Case 1.* A 38-year-old woman was complaining of fatigue, dyspnoea and vaginal bleeding for several months before admission on February 26, 1972. Seven months before this episode, she had received chloramphenicol for the second time in her life, for the treatment of bronchitis. Peripheral blood examination revealed a profound pancytopenia: haematocrit 16%, white cells 1,050 $mm^3$ (neutrophiles 160) and platelets 10,000/$mm^3$. Bone marrow biopsy showed a severely hypoplastic marrow, the cells being mainly lymphocytes. The patient was nursed in a sterile room and received androgens and transfusions for 6 weeks without improvement. She was then given horse antihuman thoracic duct lymphocyte ALG 'Berna' (total dose 150 mg/kg in 5 days). As ALG produces thrombocytopenia, its infusion was regularly followed by intensive platelet transfusion. 24 h after the last dose of ALG she was given $1.2 \times 10^{10}$ bone marrow cells ($2.3 \times 10^9$/kg) from her HL-A-semi incompatible, MLC-positive, ABO identical brother. 2 days later, she developed a general skin eruption with polyarthralgia, cervical lymphadenopathy, and severe pruritus. Abundant plasma cells were noted in the blood smears. She was thought to have serum sickness due to ALG and was started on prednisone. A few days later all symptoms gradually subsided. No signs or symptoms of GvH were observed. The change of cellular blood elements after bone marrow transfusion is illustrated in figure 1.

Reticulocyte and granulocyte counts started to rise about 15 days post grafting. The platelet count improved only slightly, but no bleeding episode was observed during the following weeks. On the 19th day after transplantation, the bone marrow aspirate revealed a marked increase in cellularity. Promyelocytes, myelocytes, numerous foci of normoblasts and occasionally megakaryocytes were found. Blood and platelet transfusions could be discontinued and the patient was discharged on June 1, 1972. During the following months she did well except for mild persisting thrombopenia with vaginal bleeding and an episode of epistaxis. These complica-

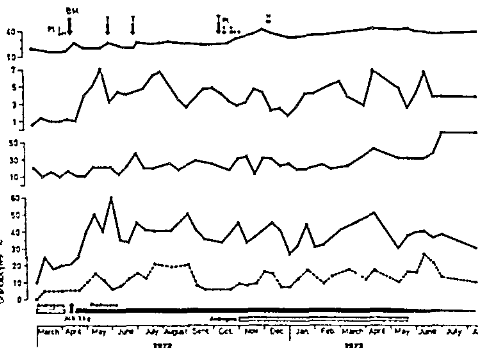



*Fig 1* Haematological events in case 1. BM = Bone marrow transfusion ($12 \times 10^{10}$ cells), Pl = platelet transfusion. T = red cell transfusion

tions were easily controlled by random platelet transfusions. In November 1972 she was restarted on androgens, and prednisone therapy was continued at very low doses (20 mg every other day). In July 1973, the patient was doing well without transfusions, and all drugs were interrupted. In August 1975, over 3 years after bone marrow transfusion, her haematocrit is normal, her granulocyte count is fluctuating around 2,000 mm³ and her platelet count is stable over 100 000 mm³. Androgens and prednisone have been discontinued over 2 years ago. Genetic marker studies, including red cell (N and $Le^b$) phaenotypes, HL-A (1, W 32, W 21) antigens, immunoglobulin groups [gm(a) antigens] and Y chromosome, revealed no evidence for engraftment. The function of the patient's T lymphocytes was studied at regular intervals during the post grafting course. As before, the patient's lymphocytes were stimulated by the donor's lymphocytes and by unrelated lymphocytes in MLC. In addition, in the cell-mediated lympholysis (CML) test [13] the patient's lymphocytes were shown, after *in vitro* sensitization, to exert a normal killing effect on $^{51}Cr$ labelled target cells carrying the incompatible HL-A antigens of the donor cells. No evidence was found for the presence of a serum factor able to block the MLC or the CML response of the patient's own or of normal lymphocytes [14]. However,

more than 1 year after bone marrow transfusion, a lymphocytotoxic antibody, specific for the incompatible donor antigen HL-A 1, was detected in her serum for the first time. This antibody has persisted since.

*Case 2.* An 8-year-old boy had recurrent respiratory infections, fatigue and an episode of epistaxis 2 weeks prior to admission to the Childrens' Hospital in Berne on January 3, 1972. Peripheral blood counts gave the following results: haemoglobin 9.1 g%, reticulocytes 0%, leucocytes 730 with 58 neutrophiles/mm³, thrombocytes 200/mm³. Bone marrow aspiration and biopsy revealed a completely aplastic marrow with only small lymphocytes and occasional plasma cells. There was no history of exposure to drugs known to be associated with AA. The patient was nursed in protective isolation and given oxymetholone, prednisone and transfusions. No improvement was seen in the subsequent 5 months, and repeated haemorrhagic and infectious episodes occurred. He progressively became refractory to random platelets, and transfusions usually were associated with febrile reactions. At that time lymphocytotoxic antibodies reactive with 55% of a lymphocyte panel were detected in his serum. ABO and HL-A typing of the family revealed the father to be the best possible marrow donor. As the patient, he had blood group A Rh positive and differed from him, by one HL-A haplotype. In addition the cross match between the patient's serum and the father's lymphocytes was very weak compared to the other family members. In the MLC test, stimulation of the patient's lymphocytes by the father's cells was weaker than with the mother's and with unrelated lymphocytes. ALG was given intravenously on 4 subsequent days (total dose 130 mg/kg). Each dose was followed by a platelet transfusion. 24 h after the last ALG infusion (June 6, 1972), $7 \times 10^9$ bone marrow cells ($2.3 \times 10^8$/kg) from his father were given intravenously. 3 weeks later some myeloid and erythroid precursor cells were detected for the first time in his bone marrow aspirate. Repeated marrow aspirations during the following 6 months revealed an increase of all elements, including megakaryocytes. Granulocyte and reticulocyte counts in the peripheral blood did not show any noteworthy increase before December 1972 (fig. 2). The platelet count was low and he still required frequent platelet transfusions.

However, after the beginning of 1973, when the patient was again on oxymetholone (1.5 mg/kg/day) and prednisone (0.3 mg/kg/day), the transfusion requirement decreased markedly. It is remarkable that after ALG and marrow infusion the patient's refractoriness to random platelets had disappeared. Except for a mild and transient HB Ag negative hepatitis, no infectious or bleeding complications occurred after marrow transfusion. No symptoms typical of GvH were observed. At present, 3 years after grafting he is back to normal activities. Over more than 1 year he has not had platelet or other transfusions. There is persistent thrombocytopenia of ±10 000/mm³ without major haemorrhagic diathesis. We have planned splenectomy in an attempt to improve this problem. The haemoglobin is normal and granulocyte counts constantly above 2,000/mm³. Genetic markers, including two red cell antigens ($Fy^b$ and $Jk^b$) and three HL-A antigens (W 10, 2 and 5) disclosed no evidence for marrow engraftment. Twenty serum globulin groups (gm) of the recipient and of the donor were identical, which prevented the detection of a B lymphocyte engraftment. MLC tests performed after transplantation showed an almost complete loss of the reactivity of the patient's lymphocytes to donor lymphocytes treated with mitomycin. In addition, the recipient's serum was found to block the response of his

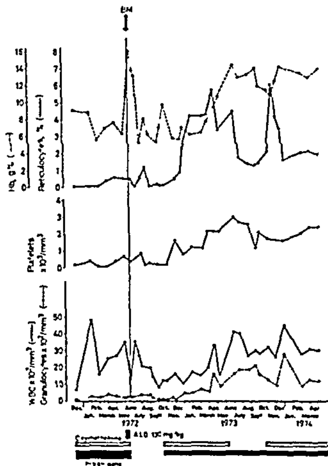


*Fig. 2* Haematological events in case 2. BM = Bone marrow transfusion.

own and of normal lymphocytes to allogeneic lymphocytes. A direct CML test [15] performed 9 months after bone marrow infusion showed no killing of donor target lymphocytes by recipient lymphocytes. In the indirect CML test, however, the patient's lymphocytes were shown to have a strong killing effect on donor cells after *in vitro* sensitization.

*Case 3.* A 12 year-old boy developed haemorrhagic diathesis in September 1970. He was first seen at the Department of Paediatrics of the University of Geneva in November 1970. In the past history there was no exposure to potentially myelotoxic agents. The haemoglobin was 6.5 g%, leucocytes, 2,600 with 550 neutrophiles per

mm³ and platelets, 27,000 mm³ Bone marrow puncture revealed a severely hypoplastic marrow with predominantly lymphoid and plasmocytoid elements

All other laboratory investigations were normal The diagnosis of idiopathic acquired aplastic anaemia was established and treatment was started with oxymetholone in a dose of 2–3 mg/kg/day and low-dose prednisone After an initial progression of the pancytopenia in the early course of 1971, a partial remission followed which persisted through 1972 The haemoglobin stabilized between 9 and 11 g%, the leucocytes, between 2,000 and 3,000 with 600–1,500 granulocytes/mm³, the platelets, around 20 000/mm³ In Winter 1973, there was clear cut evidence of a relapse which proved to be refractory to androgens and glucocorticosteroids There was haemorrhagic diathesis with profound thrombocytopenia (platelets 1 000–2,000/mm³), progressive leukopenia and granulocytopenia (granulocytes 50–150 mm³) He was given 10 platelet transfusions and a total of 20 units of whole fresh blood and packed red cells At that time, weak lymphocytotoxic antibodies reactive with 30% of a lymphocyte panel were detected in his serum On May 5, 1973, he was transferred to the University Hospital in Basel On admission he had 40 °C fever, generalized petechiae and multiple ulcerations on the oral mucosa. The pancytopenia was progressive with granulocytes of 50/mm³ Bone marrow aspiration and biopsy revealed virtual absence of haemopoetic elements HL-A and ABO typing of the family suggested the mother to be the best marrow donor

The patient was blood group A Rh negative, HL-A 2, W 16 / 2, 12. The mother was A Rh positive, HL-A 2, 12 / 1, W 18 In the patient s serum no lymphocytotoxic antibodies against the mother's lymphocytes were detectable The MLC between donor and recipient was positive The patient was nursed in a sterile laminar cross flow unit He was given sterile food Gastro-intestinal decontamination was achieved with oral nystatin and non absorbable gentamicin and Vancomycin He was conditioned with i v ALG (total dose 160 mg/kg) divided into four doses that were given as 3-hour infusions on 4 subsequent days Six platelet transfusions from 4–6 random donors were necessary to keep his platelet count above 10 000 mm³ during the conditioning period On May 21, 1973, 48 h after the last ALG $1.5 \times 10^{10}$ nucleated marrow cells from the mother were given i v ($3.8 \times 10^{8}$/kg) At the end of the marrow infusion, the patient had shaking chills and a temperature of 39 °C) Blood cultures were negative After grafting he was hospitalized for 6 weeks During that period he received only two platelet transfusions on days 8 and 15, then the platelet counts stabilized at 15 000–20 000 mm³ without haemorrhagic diathesis The white cell counts reached 2,500 with 600 granulocytes/mm³ Reticulocyte counts reached 4.3% but there was still a severe anaemia (Hct 12%) at the time of discharge on July 7, 1973 (fig 3) A bone marrow aspiration 3½ weeks after grafting revealed better cellularity, and in particular there was more activity in the myelopoesis where all stages of maturation were found Progenitor cells (CFU C and CFU E) assayed by *in vitro* culture in a methylcellulose system were virtually absent before grafting on stimulation with colony stimulating activity (CSA) and erythropoietin At 3½ weeks after transplantation they were found to be normal in quantity and quality Cytogenetic studies of the marrow cells and of peripheral lymphocytes stimulated with PHA all showed host karyotypes Red cell markers could not be used because of heavy transfusions before grafting and no immunoglobulin allotype markers were available because they were identical between donor and re-

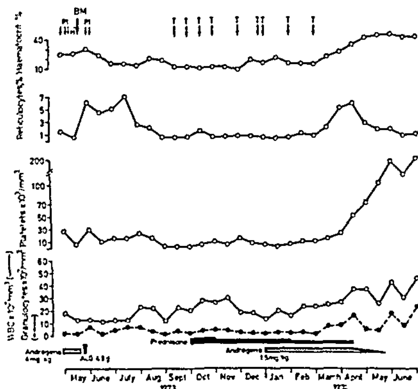


*Fig 3* Haematological events in case 3 BM = Bone marrow transfusion ($1.5 \times 10^{10}$ cells) Pl = platelet transfusion T = red cell transfusion.

cipient In October 1973 4 months after bone marrow infusion, the patient was started again on prednisone (0.5 mg/kg day) and 3 months later on oxymetholone (1.5 mg/kg day) In March 1974, his peripheral blood picture improved steadily, and androgens and prednisone were discontinued Since then, there was no more need for any transfusions At present, over 2 years after the graft, he has a normal blood picture with a haemoglobin of 15.9 g/dl, granulocytes above 2 000 mm³, and platelets around 100 000 mm³ For 18 months he has been back to all normal activities The function of the patient's T lymphocytes and B lymphocytes was studied at regular intervals after bone marrow transfusions As before grafting, the patient's lymphocytes were stimulated by donor lymphocytes in MLC The level of stimulation increased progressively

Three months after bone marrow infusion, the recipient's serum was found to block the response of his own and normal lymphocytes to allogeneic lymphocytes, a phenomenon which was not observed before At the same time, high titer lymphocy-

totoxic antibodies specific for HL A 5, W 5 and the mother's incompatible antigen W 18 were detected in the patient's serum, *demonstrating a strong immunization* against the bone marrow donor. Since blocking and cytotoxic antibodies appeared almost simultaneously, they may be closely associated. A direct CML test [15] performed 6 months after bone marrow infusion showed no killing of donor target lymphocytes by recipient lymphocytes. However, as in the second patient, in the in*direct CML test the patient's lymphocytes* were demonstrated to have a strong killing effect on donor cells after *in vitro* sensitization.

## *Discussion*

The autologous marrow restoration in these three patients suggests that treatment with ALG and HL-A non-identical bone marrow cell transfusion may be of great benefit to patients with severe AA. The mechanism of these remissions remains speculative at the moment. Spontaneous remissions appear highly unlikely because all patients were considered to have end-stage disease with extreme and long-lasting pancytopenia refractory to conventional therapy. All were polytransfused with random blood products. In the rabbit with benzene- and $^{32}$P-induced AA and in several clinical patients, split and transient haemopoietic engraftments have been achieved after conditioning with ALG followed by marrow infusion [8–12]. Despite of histocompatibility differences between donor and recipient, GvH has not been observed. Split chimaerism in the rabbit was of a rather constant pattern, donor erythrocytes, granulocytes and B lymphocytes took while T lymphocytes remained of host type [8, 9]. In rats rendered aplastic by a lethal dose of cyclophosphamide and treated with rabbit anti rat lymphocyte serum prior to the infusion of incompatible allogeneic bone marrow, no real takes were achieved [16, 17]. *However, the recipients did not develop GvH and had a significantly* prolonged survival compared to the group treated with cyclophosphamide alone. A similar beneficial and often more prolonged effect was obtained by the use of allogeneic anti-donor or anti-recipient anti-lymphocyte serum [17]. The results in our patients follow the rat model [16, 17] more closely than the rabbit [8, 9]. Despite extensive studies, no definite evidence of chimaerism could be demonstrated in our patients. Unfortunately, in the second and third one, Ig allotypes could not be used as B-cell markers because of complete identity between donor and recipient. The fact that one recipient has produced high titres of specific lymphocytotoxic antibodies against the donor, which were detectable for the first time 1

year after bone marrow infusion, implies that residual donor tissue has persisted for a while in the recipient and has then been rejected In all three patients it was demonstrated that recipient lymphocytes had a marked capacity of proliferating in MLC with donor cells, and after this phase of sensitization they showed a strong killing effect on them in the CML test. This suggests that no mutual tolerance of donor's and recipient's lymphocytes was established after bone marrow transfusion and confirms the absence of chimaerism Despite the lack of evidence of engraftments, a pure coincidence between the onset of improvement and the bone marrow graft appears highly unlikely All three patients had profound granulocytopenia and thrombocytopenia with major haemorrhagic and infectious complications, which are usually associated with a poor prognosis [1, 2, 4, 5] All had been treated with androgens, the best available supportive care for many months, two without improvement, one with only temporary benefit Moreover, in all three patients, the rise in marrow cellularity started in a similar chronological and morphological pattern that has been previously reported by other investigators [10–12]

The mechanism responsible for the demonstrated marrow repopulation by host haemopoetic cells remains obscure It is improbable that ALG alone was operative by suppressing an immune mechanism active at the level of the stem cell or the marrow matrix Auto-immune phaenomena were searched for in all patients and were all found to be negative To our knowledge, ALG treatment alone is not beneficial in the treatment of AA Moreover, ALG reduces the number of colony forming units (CFU) in mouse haemopoetic tissues both *in vivo* and *in vitro* [18] In other experimental systems, however it was shown that ALG may stimulate haemopoesis in the spleen [19] CHERTKOV *et al* [20] demonstrated in lethally irradiated mice that stimulation of haemopoetic colonization was maximal if ALG was given 24 h prior to the injection of donor haemopoetic cells It is suggested that pretreatment with ALG may be an important factor for the favourable clinical results in patients with AA given bone marrow transplantations [10–12] Our results suggest that ALG conditioning and bone marrow grafts may have a synergistic effect Ultimately a temporary lymphocyte-dependent allogeneic effect [21, 22] may have been responsible for the observed stimulation of recipient haemopoetic tissue ALG pretreatment might be an essential prerequisite for a local allogeneic effect due to its host immunosuppression allowing split engraftment without overt GvH The fact that recently two autologous marrow restorations have been observed in patients with severe aplastic

anaemia after conditioning with cyclophosphamide and grafting with *HL-A-identical sibling* marrow [23, 24] implies that other immunosuppressive regimens followed by marrow transfusion can lead to the same results as ALG *Irrespective of the underlying mechanism of the results* described, we would like to suggest that similar therapeutic trials should be attempted in patients with severe AA who do not have *HL-A-identical* sibling donors

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# Reversible Malabsorption of Folic Acid in the Elderly with Nutritional Folate Deficiency

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*Key Words* Folate deficiency Folic acid absorption Geriatric patients Malabsorption Malnutrition

*Abstract* In normal subjects the folic acid absorption (TRIFA test) was independent of age and sex Among 53 geriatric patients with nutritional folate deficiency, impaired absorption of folic acid was present in 19 (36%) After treatment with folic acid for 4 weeks the absorption returned to normal It is concluded that folate *deficiency per se can produce a malabsorption syndrome resulting in further depletion of folate* Protein deficiency and weight loss alone do not impair the absorption of folic acid

Folate deficiency due to malnutrition is not at all rare in elderly people who because of low income cannot afford a well-balanced diet, or whose dietary choice is restricted because of reduced mobility caused by physical or mental deterioration [13, 17, 19, 20] Low total caloric intake in elderly individuals with low physical activity also reduces the supply of the necessary vitamins [24], and inadequate composition of the diet may lead to depletion of the folate depots within a few months

The study reported here shows that nutritional folate deficiency *in elderly patients can reduce the ability of the intestine to absorb folic acid*

## *Material and Methods*

The 53 patients studied 11 men and 42 women, were all over 60 years (average 74 years) They were admitted to hospital for medical and social problems and had dietetic deficiencies and weight loss (>5 kg) extending over periods from 6 months

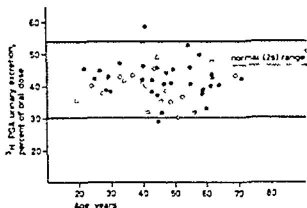



*Fig 1* Correlation between results of the TRIFA test, age and sex in 64 normal subjects ● = Male, ○ = female hatched area results obtained in the control group (2s)

to 2 years before admission. Only patients with normal renal function were included in the study

The haemoglobin concentration (normal >7.0 mmol/l) was determined by the cyanhaemoglobin method and the MCV was calculated (normal <100 fl) The serum folate (normal >9 nmol/l) and red-cell folate (normal >250 nmol/l) concentrations were determined by the *Lactobacillus casei* method [11] while the serum cobalamin concentration (normal >200 pmol/l) was determined by an isotope method [15] The formiminoglutamic acid (FIGLU) excretion was measured in 6-hour urine after histidine load (normal <20 μmol/h) Intestinal absorption of folic acid was determined by the TRIFA test [8] The control group for the TRIFA test consisted of 64 randomly selected non folate-deficient subjects aged 23–70 years. Normally more than 30% of the orally administered dose is excreted in the ensuing 24-hour urine

*Results*

The absorption of folic acid as assessed by the TRIFA test was found to be independent of age and sex in the control group (fig 1)

In the patient group the serum folate concentrations ranged from 0.5 to 9.0 nmol/l (mean 2.8 ± 2.2), while the red-cell folate concentrations varied between 22 and 221 nmol/l (mean 114 ± 67) The FIGLU excretion ranged from 4 to 190 μmol/h (mean 58 ± 50) Haemoglobin levels below 7.0 mmol/l were seen in 25 patients, the MCV was increased in 30 patients The serum cobalamin concentration was normal in all patients

*Table I.* Causes of dietary insufficiency in 53 patients with nutritional folate deficiency

| Causes of dietary insufficiency | Males | Females |
|---|---|---|
| Faulty dietary habits | | 6 |
| Abuse of alcohol and medicine | 1 | 5 |
| Forgetfulness, confusion | 1 | 6 |
| Insteady gait (cerebral strokes) | | 5 |
| Tooth problems | | 2 |
| Anorexia, neurasthenia | 5 | 4 |
| Loneliness, improper cooking | 3 | 6 |
| Difficulties of swallowing | | 2 |
| Hyperthyreosis | | 4 |
| Undiscovered | 1 | 2 |
| Total | 11 | 42 |

*Table II.* Absorption of folic acid (TRIFA test) after a 10-day zero-calorie diet

| Sex | Age, years | Weight loss, kg | TRIFA, % |
|---|---|---|---|
| M | 30 | 10.0 | 32.0 |
| F | 29 | 6.8 | 42.0 |
| F | 33 | 6.7 | 51.8 |
| F | 26 | 7.2 | 43.0 |
| F | 37 | 8.2 | 52.0 |

except 3, in whom the values were 35, 120 and 150 pmol/l, respectively. None of these patients had pernicious anaemia as assessed by the Schilling test and intrinsic-factor determination in the gastric secretion. Bone marrow studies were performed in 21 patients. Dysplasia of the granulopoiesis as a sign of folate deficiency [1] was demonstrated in 15 patients.

In most of the patients, the cause of the dietary insufficiency could be detected, in some cases, by information given by relatives (table I). Only in 3 patients with distinct evidence of dietary insufficiency was it impossible to trace the cause. In some cases, the patients had placed themselves on an unbalanced diet in the belief that this would have a beneficial effect on colitis, and the caloric requirements were mainly covered by the ingestion of alcohol in others. Owing to confusion and cerebral arteriosclerosis, some patients simply forgot to have their principal meals. In other

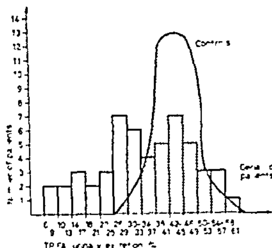


*Fig 2* Histogram showing the distribution of the absorption of folic acid (TRIFA test) in 53 patients with nutritional folate deficiency Hatched area = results obtained in the control group (2 s)

cases, severe physical handicaps restricted the patients' possibilities of doing their shopping of food Often, anorexia had developed in association with a fortuitous infectious disease and had persisted for many months About 20% of the patients were solitary and lived on an unbalanced diet consisting of bread, biscuits and coffee In 4 patients, the weight loss and anorexia were due to thyreotoxicosis.

Determinations of the absorption of folic acid performed by the TRIFA test within the first 3 weeks in hospital gave values below the lower normal limit in 19 patients (fig 2) In the group as a whole, 34 ± 13% of the oral dose of folic acid was excreted in the 24 hour urine, which is significantly different from the values observed in the control group ($p<0.01$)

Having been subjected to the TRIFA test, all patients were treated with an oral dose of 5 mg folic acid three times daily In 11 patients who all showed low absorption of folic acid initially, the TRIFA test was repeated after treatment for 1 month Figure 3 shows that in 9 of these patients, the test then revealed normal values, and in the remaining 2 the absorption had improved although it had not reached the normal level The difference in absorption is significant at the 0 95 level ($p<0.0005$)

to certain organs, as has been observed in conditions with folate deficiency [9, 12, 21, 22]

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# Proliferative Activity of Undifferentiated Cells (Blast Cells) in Preleukaemia

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*Key Words.* Autoradiography · Blast cells · Cytophotometry · DNA synthesis · Preleukaemia

*Abstract.* Undifferentiated cells (blast cells) in preleukaemic states show labelling indices comparable to those of leukaemic blast cells. Since this cell population can remain constant over months or even years, a steady state must exist between the production of blast cells and cell death. It is more likely, however, that in preleukaemic conditions blast cells may differentiate to morphologic 'normal' haemopoietic cells. The fact that the latter show an impaired cell proliferation supports the view that they derive from an affected stem cell compartment.

Undifferentiated cells (blast cells) can occur in the bone marrow, more seldom in the bone marrow and blood of patients with bicytopenia or pancytopenia. The number of blast cells can remain constant over several months or even years. They differ in this from the blast cells of acute leukaemia, the number of which increases continuously.

Leukaemic blasts have, as opposed to normal haemopoiesis, a reduced proliferative activity and a prolonged generation time [2, 3, 5, 7, 8, 10–12, 14]. Their labelling index is variable and, for example, dependent on the degree of the leukaemic infiltration [3, 8, 12, 14]. The less the density of the blast cells in the marrow, the higher is the labelling index in general. Accordingly, in a so-called smoldering acute leukaemia a higher labelling index was found than in acute myeloblastic leukaemia [13].

[1] We wish to thank Miss M. Broszies for her excellent technical assistance.

In our investigations on ineffective haemopoiesis in preleukaemic states [4, and unpub.] we also examined the small blast cell population, specifically with the question whether blast cells in preleukaemia always show a higher labelling index, corresponding to the slight marrow infiltration, than leukaemic blast cells.

## Methods

Single cell suspensions of bone marrow were incubated for 1 h at room temperature with tritiated thymidine (2 µCi/ml; specific activity 5 Ci/mM). Smears were stained by the May-Grünwald-Giemsa method. Cells were photographed and the cell areas marked with an 'Objektmarker' (Leitz, FRG) on a second slide. The panchromatic stain was leached out and then the smears were restained by the Feulgen method (hydrolysis in 1 N HCl at 60 °C for 9 min). The DNA content was measured with a cytophotometer UMSP I (Zeiss, FRG) at 560 nm. Autoradiographs were prepared by the dipping method using the emulsion K5 (Ilford, England) as described previously [4].

## Results

The undifferentiated cells (blast cells) of the bone marrow of 5 patients were examined. The most important haematologic data are summarized in table I. In the first examination, all patients had pancytopenia or bicytopenia with normal or increased cell density of the bone marrow. The percentage of blast cells of the bone marrow accounted for maximally 15%. In the peripheral blood blast cells were only detectable in two patients. In three of them, the complete picture of an acute leukaemia appeared after a few months.

The labelling index of the blast cells (table II) was 4.5–10% in 4 of 5 cases, only in 1 case was the index very low with 2%. In comparison to the number of blast cells in DNA synthesis, a relatively high number of unlabelled cells has a DNA content between 2 c and 4 c (U cells).

## Discussion

The labelling indices of the undifferentiated cells (blast cells) of preleukaemia are comparable to those of leukaemic blasts [5, 7, 8, 10–12]. It has, however, been shown that the percentage of DNA synthesis of leukaemic blasts is smaller the denser the leukaemic marrow infiltration is at

*Table 1* Main haematological findings in the patients examined

| se | Age years | Hb g/100 ml | Neutrophils/µl | Platelets $\times 10^3$/µl | Blast cells/µl blood | Blast cells in bone marrow,*% | Ratio E G | Diagnosis | Course |
|---|---|---|---|---|---|---|---|---|---|
| | 54 | 12 0 | 210 | 16 | 0 | 4 | 1 0 | leukopenia and thrombopenia | haematolog c ta unchanged months) Bact infections |
| | 48 | 8 7 | 2 740 | 97 | 571 | 15 | 0 2 | smoldering acute leukaemia? | died 6 month diagnosis AM |
| | 59 | 6 5 | 1 110 | 120 | 0 | 6 | 0 25 | anaemia and leu kopenia hyperplastic bone marrow (Preleukaemia?) | died 4 months diagnosis AM |
| | 23 | 5 2 | 384 | 17 | 24 | 7 | 1 0 | pancytopenia with hyperplastic bone marrow Early stage of AML? | blood transfu Haematolog c ta unchanged (6 months) |
| | 33 | 8 5 | 360 | 87 | 0 | 10 | 0 4 | pancytopenia with hyperplastic bone marrow (Preleukaemia?) | died 3 months diagnosis AMI |

Ratio E G = Ratio erythropoiesis myelopoiesis The percentage of the blast cells refers to all the ce bone marrow in the assessment of 200 cells

the time of the examination [8, 12, 14] One would therefore expect more blast cells in DNA synthesis in our patients than in cases with dense leukaemic infiltration In spite of the small number of blast cells in our 5 patients, their labelling index is low A further difference to the blasts of acute myeloblastic leukaemia is the slight or even absent tendency to increase in numbers Many patients with the diagnosis 'preleukaemia' or smoldering acute leukaemia' can have a constant number of blast cells in the bone marrow over many months or even years It is not possible to give an exact explanation of this phenomenon The following hypotheses present themselves (1) the lifetime of the blast cells formed is short, they die intra medullarly Production and death of blast cells are therefore, in a steady state, (2) blast cells can differentiate to cells of the 'normal' hae

*Table II* Results of the cytophotometric-autoradiographic examination

| Case No | n | $G_1$ | S | $G_2$ | U |
|---|---|---|---|---|---|
| 1 | 153 | 81 6 | 8 5 | 0 7 | 9 2 |
| 2 | 209 | 91 4 | 4.3 | 0 5 | 3 8 |
| 3 | 202 | 88 1 | 9 9 | 1 0 | 1 0 |
| 4 | 89 | 85.4 | 10 1 | 1 1 | 3 4 |
| 5 | 193 | 97 0 | 2.0 | 0 | 2.0 |

n = Number of cells examined U = unknown, unlabelled cells with DNA values between the diploid and tetraploid content

mopoiesis [6, 9] This seems to be possible, at least from the morphological point of view, because the 'normal haemopoiesis and, above all, the myelopoiesis of our patients show abnormalities with numerous transitional forms between blast cells and promyelocytes or myelocytes Our cytophotometric autoradiographic investigations on myelopoiesis of these cases show a highly disturbed proliferation with a decreased labelling index and a high percentage of cells in the presynthetic phase (unpublished results) For example in patient 5, 98% of all promyelocytes and myelocytes are found in the $G_1$-phase and only 2% had incorporated $^3H$ thymidine These cells do not differ in this in their proliferative activity from the blast cells We might, therefore, assume that the morphologically undifferentiated cells (blast cells) are progenitors of haemopoiesis or of a part of haemopoiesis [9] This hypothesis is supported by the finding of HOELZER *et al* [6] that myeloblasts may differentiate in *diffusion chambers* Furthermore, the demonstration of the same chromosomal aberrations in blast cells and erythroblasts in cases of acute leukaemia shows that 'normal erythropoiesis is also affected [1] In preleukaemic states the reduced proliferative activity of the haemopoiesis indicates that *normal myelopoiesis and erythropoiesis* derive from an affected stem cell compartment

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|---|---|---|---|---|---|---|---|---|---|
| 1 | 54 | 12.0 | 210 | 16 | 0 | 4 | 1.0 | leukopenia and thrombopenia | haemato ta uncha months) infection |
| 2 | 48 | 8.7 | 2,740 | 97 | 571 | 15 | 0.2 | smoldering acute leukaemia? | died 6 m diagnosis |
| 3 | 59 | 6.5 | 1,110 | 120 | 0 | 6 | 0.25 | anaemia and leukopenia, hyperplastic bone marrow (Preleukaemia?) | died 4 m diagnosis |
| 4 | 23 | 5.2 | 384 | 17 | 24 | 7 | 1.0 | pancytopenia with hyperplastic bone marrow. Early stage of AML? | blood tr Haemato ta unchai (6 month |
| 5 | 33 | 8.5 | 360 | 87 | 0 | 10 | 0.4 | pancytopenia with hyperplastic bone marrow (Preleukaemia?) | died 3 m diagnosis |

*Ratio E:G = Ratio erythropoiesis: myelopoiesis. The percentage of the blast cells refers to all* the bone marrow in the assessment of 200 cells.

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| 3 | 202 | 88 1 | 9 9 | 1 0 | 1 0 |
| 4 | 89 | 85 4 | 10 1 | 1 1 | 3 4 |
| 5 | 193 | 97 0 | 2 0 | 0 | 2 0 |

n = Number of cells examined, U = unknown, unlabelled cells with DNA values between the diploid and tetraploid content

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# Low Mitotic Activity in Eosinophilic Leukaemia

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*Key Words* Bone marrow eosinophils Eosinophilia Eosinophilic leukaemia Leukaemia Mitotic index

*Abstract* The mitotic indices of the eosinophilic precursors in the bone marrow of 3 untreated patients with eosinophilic leukaemia were found to be significantly lower than those of 11 patients with reactive eosinophilia and those of 15 healthy controls The same differences were found in the neutrophilic precursors and in the myeloblasts indicating leukaemic involvement of both the eosinophilic and neutrophilic cell lines Mitotic countings therefore seem to be of diagnostic significance in this rare disease

The separation of eosinophilic leukaemia (EL) from other conditions with eosinophilia is difficult. Furthermore, there are at least two different types of EL (a) acute EL with a marked number of myeloblasts (MB) in the peripheral blood and immature eosinophils dominating in the bone marrow (patients K S and E R, in this paper), and (b) chronic EL with increased numbers of mostly mature eosinophils in the blood (patient T M) [3 7, 9, 10, 19] Although it is quite clear that leukaemic cells are rather heterogenous with respect to their kinetic behaviour, low mitotic indices of the granulopoietic precursor cells, especially the MB, indicate leukaemic involvement of these cells Thus, significantly lower indices have been found in acute myeloid leukaemia [6, 15, 28, 29] and in chronic myeloid leukaemia especially in the more advanced cases [2, 5, 21, 26–28]

The aim of the present work was to investigate whether any characteristic changes in mitotic activity of the MB, the neutrophilic precursors

and the eosinophilic precursors will occur in cases of EL, thus, making the diagnosis surer

## Material and Methods

*Patients* Three patients with EL, 11 patients with reactive eosinophilia (RE) and 15 haematologically healthy persons were included in this study None of the patients had received steroids or cytostatics

*EL group* The first patient, T M., a 52 year old man was studied on December 4, 1974 Hb was 5 7 g/dl, WBC 24 300 with 72% mature eosinophils and 2% MB thrombocytes 162,000 The patient had splenomegaly and increasing WBC Thus, on March 25, 1975 the WBC were 183 000 with 88% eosinophils The LAP score was negative A detailed case report with a chromosome analysis is given by MITTELMAN *et al* [18] The chromosome study revealed no Ph¹-chromosome but an isochromosome 17 in all bone marrow metaphases The patient is still alive

The second patient K S, a 65 year old man, was studied on June 27, 1972 Hb was 4 9 g/dl WBC 32,500 with 2% immature eosinophils 41% MB and 14% myelocytes thrombocytes only 1 000 VAMP treatment was not successful and the patient died on November 11, 1972 Autopsy was not performed

The third patient E R a 61 year old woman was studied on April 4 1974 Hb was 8 3 g/dl WBC 15 500 with 4% immature eosinophils and 55% MB thrombocytes 35 000 Treatment with Cytosine arabinoside and Rubidomucin has been rather successful and the patient is alive Bone marrow data for the patients above are given in table I

*RE group* 11 patients with diagnoses such as asthma allergic reaction helminthic diseases Hodgkin s disease and dermatitis herpetiformis were studied The bone marrow eosinophils comprised 6 4–39.3% of all nucleated cells and in the peripheral blood 29–64% were eosinophils Hb was 10.5–14.2 g/dl and WBC 4 600–28 600

*Normals* 15 probands without perceivable haematologic disorders and with clinical diagnoses such as spondylolisthesis cervical disc prolapse adipositas psychiatric disorders and unverified hypogonadismus served as controls Hb 12.1–16 6 g/dl and WBC 2,200–8,300 In the bone marrow the eosinophils comprised 1 4–5 1%

*Bone marrow examination* The bone marrow smears were stained with May Grünwald Giemsa Through examination of 1 000–2,500 (mean 1 094) nucleated cells the percentages of granulocytic cells at different stages of maturation were determined The cells were classified according to HEILMEYER and BEGEMANN [13] and SJÖGREN [26] A cell was considered to be in mitosis from the stage of partial loss of nuclear membrane in prophase until nuclear reconstitution had appeared at the end of the telophase The granulocytic precursor cells and their mitoses are described below

*Myeloblasts* The diameter varies from 12 to 20 μm but sometimes smaller ones micromyeloblasts, are found. The nucleus with 2–6 light blue nucleoli is round or oval and with finer chromatin than the pronormoblasts. The nucleus occupies most of the cell and the nuclear border is distinctly but delicately drawn There are no

*Table I* Bone marrow data from 3 patients with EL (values give percentage of all nucleated cells)

| Patient | MB | NPMC | NMC | EPMC | EMC | Eosinophils at all | Remarks |
|---|---|---|---|---|---|---|---|
| T M | 2.2 | 5 4 | 13 6 | 11 6 | 18 9 | 53 9 | 'nuclear flags', mixed baso-eosinophilic granules |
| K S | 16 2 | 19 4 | 4 9 | 4 0 | 10 8 | 34 7 | vacuoles in the cytoplasm mixed baso-eosinophilic granules |
| E. R | 22.7 | 3 5 | 0 4 | 15 3 | 17 7 | 57 7 | |

granules in the narrow basophilic cytoplasm In the mitotic phase the chromosome formation occupies most of the cell

*Neutrophilic (NPMC) and eosinophilic (EPMC) promyelocytes* Diameter 18–25 μm The nucleus is large with visible nucleoli. The cytoplasm is basophilic The granules of the NPMC are large and azurophilic The EPMC have a mixture of azurophilic and eosinophilic granules and sometimes there are large violet granules too especially in the leukaemia cases The chromosomes are similar to those in the MB

*Neutrophilic (NMC) and eosinophilic (EMC) myelocytes* The cells are smaller than in the PMC (14–20 μm) The nucleus with a coarse chromatin structure and no nucleoli is rounded or kidney-shaped and is often situated eccentrically The granulation of the cytoplasm is specifically neutrophilic or eosinophilic The mitotic figures are comparatively small with thick chromosomes.

*Mitotic Indices*

*EL group* In the 3 patients, 858 11 042 and 4,500 MB respectively were counted and 6 33 and 56 mitoses respectively, were found 6 900 17 040 and 1 150 NPMC+NMC respectively were counted and 31 62 and 6 mitoses respectively were found 10 903 10 088 and 6,540 EPMC+EMC respectively, were counted and 83 44 and 36 mitoses respectively were found

*RE group* 190–1 261 (totally 7 481) MB were counted and 5–27 (totally 161) mitoses were found 1 476–7,890 (totally 52,326) NPMC+NMC were counted and 20–101 (totally 613) mitoses were found. 1 040–5 016 (totally 28,254) EPMC+EMC were counted and 13–67 (totally 358) mitoses were found

*Normals* 33–191 (totally 1 137) MB were counted and 27 mitoses were found. 1 316–3 614 (totally 30 448) NPMC+NMC were counted and 13–43 (totally 348) mitoses were found 17 214 (totally 1,576) EPMC+EMC were counted and 19 mitoses were found

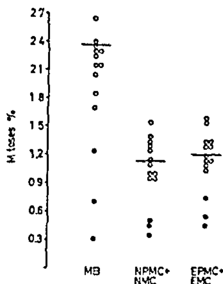



*Fig 1* Separate mitotic indices of MB, NPMC+NMC and EPMC+EMC in 3 patients with EL (●) and in 11 patients with RE (O) Horizontal lines indicate mean value of 15 normals

Because of the nuclear and cytoplasmatic asynchrony of neutrophilic and eosinophilic precursors in the EL group it was very difficult to separate the promyelocytic and myelocytic mitoses. Therefore the PMC and MC were pooled into one group

## *Results*

The mitotic indices of the MB in the EL group were significantly lower than those of the MB in the RE group (Mann-Whitney two-tailed U test, $p<0.02$) (fig 1) The mitotic indices of the NPMC+NMC in the EL group were significantly lower ($p<0.02$) than those of the corresponding cells in the RE group The mitotic indices of the EPMC+EMC in the EL group were significantly lower than those of the EPMC+EMC in the RE group ($p<0.02$) (fig 1) In the EL group 12–27% of the eosinophils were EPMC and 31–35% EMC In the RE group 5–11% (median 7) were EPMC and 18–45% (median 28) were EMC. In the normals 3–12% (median 5) were EPMC and 26–43% (median 34) were EMC There is a significantly higher proportion of EPMC in the leukaemia group compared to the RE group and to the normals The proportions of eosinophilic precursors (EPMC+EMC) within the eosinophilic pool of the RE patients

and the normals are in good agreement with data by GROSS and GEDIGK [11] who found 37% EPMC+EMC in normal subjects

*Discussion*

There has always been some controversy regarding the classification of EL in the group of true leukaemias and some authors have considered it doubtful that this rare disease is a clinical entity [4, 20, 22, 31, 33] Various abnormalities of the eosinophils in EL have been recorded such as nuclear-cytoplasmatic asynchrony, abnormally large eosinophilic granules, mixed basoeosinophilic granules and vacuoles in the cytoplasm [1, 3, 7, 20, 30, 32, 33], but there is no proof that similar changes do not occur in dieseases with benign eosinophilia. Karyotype analyses have shown chromosome abnormalities in some cases of EL [8, 10, 12, 14, 17, 18, 25] but only in a very few cases the aberrations can be referred to certain cell types, e g both eosinophils and neutrophils [12, 17, 18] Leukaemic involvement of both these cell lines has also been proposed in morphologic and histochemical investigations [1, 19] If we accept the idea of the MB as the precursor of both the eosinophils and the neutrophils it may be useful to study the kinetic behaviour of the MB separately As mentioned before low mitotic indices of the MB is probably pathognomonic for the myeloid leukaemias Separate mitotic countings of the NPMC+NMC and the EPMC+EMC would give information of the extension of the leukaemic cell population as well Low mitotic indices of the EPMC+EMC in combination with dominance of morphologically abnormal eosinophils in the bone marrow may then justify the diagnosis of EL. All the 3 patients reported here fulfilled these criterias The median values for the mitotic indices of the EPMC+EMC were 0.55% in the EL group and 1 27% in the RE group This difference cannot be the result of various proportions of PMC in the two groups since there were more PMC, normally known to have higher mitotic indices than the MC [16, 23, 24] in the EL group Very little is known about the mitotic activity of the eosinophils in various conditions with eosinophilia A very high mitotic index of 2.3% of the EPMC+EMC is reported by GROSS and GEDIGK [11] and in two cases of chronic myeloid leukaemia the corresponding indices were 1 24 and 1 39% respectively, i e the same as those of the NPMC+NMC [2] In this paper the mitotic indices of the eosinophils were slightly higher than those of the neutrophils in all 3 patient groups Thus the mitotic

activity of the EPMC+EMC in cases of RE and in normals could be estimated to be 1 0–1 6% and significantly lower in cases of EL.

*Acknowledgement* I wish to thank Dr MAJ LUNDSTRÖM, of Stockholm who brought the third EL patient to my attention and supplied me with bone marrow smears

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# Receptor Studies on 19 Cases of Non-Hodgkin Malignant Lymphocytic Lymphoma

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*Key Words* Antibodies Cell receptors Immunology Lymphocytes Malignant lymphoma

*Abstract* The receptor profile of 19 cases of non Hodgkin lymphocytic lymphoma has been described In nodular lymphomas, the values commonly fall within normal limits In 2 cases, the nodules separated from the paranodular tissue contained a proportion of sheep-erythrocyte-rosetting cells as well as fluorescent cells The diffuse lymphomas show predominance of fluorescent cells but some contain numerous B and T cells These represent 'co-operative' neoplasms In 3 cases, a receptor profile characterized by high frequency of surface fluorescence and a low incidence of IgG and EAG receptors was noted, this may represent a pattern for a new subclass of malignant lymphoma Tumour cells rich in surface fluorescence contained abundant ribosomes. Receptor silence was noted only in a single case

Current attempts to understand the nature of lymphoid neoplasms postulate that these tumours represent an abnormal immunological response The introduction of antigen is normally followed by a proliferation of macrophages and various populations of lymphocytes Early stages of this response are characterized by the participation of antigen handling cells and later by effector cells which either synthesize Ig or are agents of cell mediated immunity At critical points in this cellular sequence the reacting system may be frozen, for reasons unknown, in attitudes of continued cellular proliferation [8] The presence of recognisable receptors on the surface of neoplastic cells has facilitated the study of the cellular composition of lymphoid tumours Thus a malignant mediastinal lymphoma (Sternberg sarcoma) has been shown as thymic in origin [9], follicular lymphomas have a B cell origin [4] and 10 cases of non-Hodgkin lymphoma have been classified as

B or T cell neoplasms [7] The present report attempts to correlate morphology with functional studies in a series of 19 cases of non-Hodgkin malignant lymphocytic lymphoma

## *Materials and Methods*

Lymph nodes were collected fresh at operation, and immediately transferred to Hank's balanced salt solution, or medium 199 in a sterile container at room temperature The node and container were then placed in melting ice for conveyance to the laboratory On arrival, nodes were sectioned into 2 mm slices, and a portion fixed for histology The remaining node was minced into small fragments, or teased in medium 199 containing heparin 5 units/ml, to produce a cell suspension The suspension was filtered through 80-mesh stainless steel gauze to remove cell clumps Viability was assessed by trypan blue dye exclusion, and smears of the suspension were made The cell suspension was washed 3 times in medium 199, and cells were counted

*Surface receptor determination* The techniques employed here have been reported in detail elsewhere [5]

*Sheep-erythrocyte-rosetting cells* Washed fresh red blood cells from a single sheep were used throughout Lymph node cells and sheep red blood cells were mixed in a ratio of 1 40, incubated for 5 min at 37°C, centrifuged at 120 *g* for 5 min and incubated for 2 h at 4°C Serum-free medium 199 was used throughout Rosettes were suspended by gentle rotation of tubes in the long axis, sealed under a coverslip and counted wet By this method 50–60% of normal peripheral blood mononuclears form rosettes with more than 3 erythrocytes

*Receptors for immune complex (erythrocyte + antibody IgG EAIgG)* The test particle was the sheep erythrocyte coated with a high subagglutinating dose of rabbit anti-sheep erythrocyte IgG Lymph node cells and sensitized sheep erythrocytes were mixed in a ratio of 1 40, incubated for 10 min at 20°C, centrifuged at 120 *g* for 5 min, and incubated for 2 h at 4°C. Rosettes were resuspended by vigorous end-over-end rotation of the tube

*Receptors for complement (erythrocyte + antibody + complement EAC)* Sheep erythrocytes were sensitized by incubation with a high subagglutinating dose of rabbit anti-sheep erythrocyte IgM antibody for 1 h at 37°C. They were then treated with fresh human serum or guinea pig complement (Wellcome) using a two-stage procedure previously described [5] The presence of complement on the sensitized cells was shown by their agglutinability with anti-C3 antiserum (Meloy) they were not agglutinated by anti-C4 Rosettes were prepared by mixing lymph node cells with sensitized cells in a ratio of 1 40 incubating for 10 min at 37°C. They were then centrifuged at 120 *g* for 5 min and incubated for 2 h at 4°C Rosettes were suspended by vigorous end-over-end rotation of the tube Antibody sensitized cells alone gave low background rosettes with normal peripheral blood lymphocytes (0.2–4 4%) and similar numbers of rosettes were obtained at 4°C incubation (mean value 23%) as at 37°C (mean value 21%) with complement-sensitized cells These cells formed rosettes with most surface Ig+ lymphocytes, and with a high proportion of glass-adherent cells from normal peripheral blood

*Neutral red test* Phagocytic cells were detected by their characteristic pattern of con

centration of neutral red dye when incubated in dilute solution of dye in phosphate-buffered saline

*Surface-Ig-carrying cells* Cells carrying surface Ig were detected by a sandwich technique using polyvalent rabbit anti human Ig antiserum, followed by reaction with goat anti rabbit antiserum coupled with fluorescein isothiocyanate (FITC conjugate) Aggregates were removed by high-speed centrifugation before use. All viable cells showing detectable surface fluorescence were counted Fluorescent capping cells showed clear caps, or confluent ring fluorescence, fluorescent non-capping cells were classified as mononuclear cells showing small discrete dots of fluorescence diffusely localized on the cell surface

*Separation of nodules from nodular lymphomas* Nodules were separated from the spleen of case 6, and a lymph node from case 9 In both cases the nodules were 'washed' free of loosely adherent cells by threefold sedimentation through medium 199 at 1 *g* The nodules were then disrupted by squeezing through 80-mesh stainless steel gauze

*Histology* The tumours were classified as nodular or diffuse, and the degree of differentiation assessed in terms of cell size, nuclear regularity, amount of cytoplasm and prominence of nucleoli The Rappaport classification was used but in the diffuse lymphomas a need was felt for a category of 'intermediate differentiation' [1] Cases 1, 2, 3, 6, 7 in the diffuse lymphomas were classed as poorly differentiated, the remained as intermediate The nodular lymphomas correspond to the Rappaport poorly differentiated type Histiocytic lymphomas were rigorously excluded from both series

## *Results*

Lymph nodes removed from patients without signs of lymphoreticular disease showed a wide variation in receptor pattern (table I) Total surface-Ig-positive cells never exceeded 50% and the mean number was 29%. Higher values were recorded for T cells which gave a mean of 43% Proportions of cells bearing receptors for IgG or complement-sensitized erythrocytes bore no constant relationship either to immunofluorescent cells (B cells) or sheep-erythrocyte-rosetting cells (T cells) in lymph node cell suspensions

The three pathological but non-neoplastic nodes gave unusual values (table II) Case 1 was from a patient with toxoplasmosis and histology showed numerous large germinal centres A high proportion of surface-Ig-positive cells was present, the number of IgG- and EAC-rosetting cells was relatively low Case 2 was a lipomelanic reticulosis containing an excess of plasma cells, about 30% 'receptor-silent' cells were present Case 3 was a chronic non specific lymphadenitis from a patient with dermatitis Marked paracortical hyperplasia was present and the percentage of T lymphocytes was increased

*Table I* Lymph nodes from 10 cases without overt lymph node disease

| Case No | Viability % | SRBC rosettes % | IgG rosettes % | EAC rosettes % | Fluorescent capping cells % | Fluorescent non-capping cells % | Fluorescent total cells % | Neutral red phagocytes % |
|---|---|---|---|---|---|---|---|---|
| 1 | 80 | 59 | 26 | 15 | 29 | 5 | 34 | 5 |
| 2 | 85 | 48 | 56 | 36 | 13 | 3 | 16 | 20 |
| 3 | 82 | 41 | 8 | – | 14 | 8 | 22 | – |
| 4 | 87 | 24 | 12 | – | 30 | 8 | 38 | – |
| 5 | 80 | 38 | 13 | 52 | 26 | 9 | 35 | 27 |
| 6 | 65 | 55 | 18 | 40 | 28 | 4 | 32 | 27 |
| 7 | 84 | 51 | 7 | 5 | 8 | 4 | 12 | 11 |
| 8 | 74 | 35 | 29 | 33 | 32 | 2 | 34 | 7 |
| 9 | 86 | 32 | 11 | 24 | 10 | 38 | 48 | 16 |
| 10 | 93 | 50 | 45 | – | 13 | 9 | 22 | – |
| Mean ± SD | 82±8 | 43±11 | 23±17 | 29±16 | 20±9 | 9±11 | 29±11 | 16±9 |
| Range | 65–93 | 24–59 | 7–56 | 5–52 | 8–32 | 2 38 | 12–48 | 5–27 |

Viab l ty is expressed as a percentage of viable cells in the lymph node suspension The different classes of lymph node cells are also expressed as a percentage of cells in suspens on.

*Table II* Lymph nodes from 3 cases with non-neoplastic lymphadenopathy

| Case No | Diagnosis | Viabil ty % | SRBC rosettes % | IgG rosettes % | EAC rosettes % | Fluorescent capping cells % | Fluorescent non-capping cells % | Fluorescent total cells % | Neutral red phagocytes % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Toxoplasmosis | 78 | 15 | 7 | 2 | 47 | 3 | 50 | 7 |
| 2 | Lipomelanic reticulosis | 90 | 34 | 6 | 6 | 18 | 3 | 21 | 9 |
| 3 | Non-specific lymphaden tis | 68 | 52 | 9 | ND | 16 | 5 | 21 | 8 |
| Mean ± SD | | 79±11 | 35±17 | 7±2 | 4±3 | 27±17 | 4±1 | 31±17 | 8±1 |
| Range | | 68 90 | 18 52 | 6–9 | 2–6 | 16–47 | 3 5 | 21 50 | 7–9 |

*Table III* Lymph nodes from 8 cases of nodular lymphocytic lymphoma

| Case No | Viability % | SRBC rosettes % | IgG rosettes % | EAC rosettes % | Fluorescent capping cells % | Fluorescent non-capping cells % | Fluorescent total cells % | Neutral red phagocytes % |
|---|---|---|---|---|---|---|---|---|
| 1 | 90 | 59 | 9 | 12 | – | – | – | 5 |
| 2 | 90 | 52 | 41 | – | 31 | 10 | 41 | 6 |
| 3 | 80 | 48 | 23 | 29 | 23 | 12 | 35 | 1 |
| 4 | 71 | 41 | 21 | 44 | 30 | 25 | 55 | 2 |
| 5 | 73 | 30 | 20 | 15 | 35 | 4 | 39 | 8 |
| 6 spleen | 90 | 42 | 38 | 27 | 32 | 10 | 42 | 4 |
| 7 | 69 | 8 | 6 | 4 | 38 | 27 | 65 | 17 |
| 8 | 75 | 46 | 67 | 8 | 31 | 9 | 40 | 6 |
| Mean ± SD | 80±9 | 41±16 | 28±20 | 20±14 | 31±5 | 14±9 | 45±11 | 6±5 |
| Range | 69–90 | 8–59 | 6–67 | 4–44 | 23–38 | 4–27 | 35–65 | 1–17 |

*Table IV* R supraclavicular node of nodular lymphoma case 9

| | Paranodular tissue % | Nodule, % |
|---|---|---|
| SRBC rosettes | 27 | 21 |
| EAIgG rosettes | 25 | 22 |
| EAC rosettes | 32 | 5 |
| Neutral red phagocytes | 1 | 2 |
| Fluorescent capping cells | 14 | 26 |
| Fluorescent non-capping cells | 57 | 64 |

Viability nodule 80% cells viable paranodular tissue 76 5% cells viable

The results in 8 cases of nodular lymphocytic lymphoma (table III) show the majority of cases have a receptor pattern within normal limits Case 6 underwent a staging laparotomy, the spleen contained multiple small lymphomatous nodules in which both sheep-erythrocyte rosetting cells and fluorescent cells were present in approximately equal numbers Case 7 had a preponderance of fluorescent cells and unusually low percentages of IgG and EAC-rosetting cells Case 8 had a high percentage of IgG rosettes suggesting that this receptor can be expressed both by T and B cells Case 9

*Table V* Lymph nodes from 11 cases of diffuse lymphocytic lymphoma

| Case No | Viability % | SRBC rosettes % | IgG rosettes % | EAC rosettes % | Fluorescent capping cells % | Fluorescent non-capping cells % | Fluorescent total cells % | Neutral red phagocytes % |
|---|---|---|---|---|---|---|---|---|
| 1 | 84 | 28 | 23 | 17 | 57 | 37 | 94 | 6 |
| 2 | 83 | 8 | 6 | 3 | | | 74 | 1 |
| 3 | 88 | 6 | 55 | – | 36 | 34 | 70 | 2 |
| 4 | 80 | 14 | | – | 47 | 17 | 59 | – |
| 5 | 93 | 14 | 4 | 4 | | | 53 | 1 |
| 6 | 91 | 28 | 58 | 50 | 15 | 27 | 42 | 18 |
| 7 | 85 | 64 | 11 | 12 | | | 3 | 4 |
| 8 | 81 | 38 | 23 | 22 | 3 | 30 | 33 | 4 |
| 9 | 90 | 40 | 40 | | 43 | 7 | 50 | – |
| 10 | | 14 | 17 | 21 | 1 | 2 | 3 | 3 |
| 11 | 62 | 26 | 8 | 7 | 38 | 2 | 40 | 2 |
| Mean ± SD | 84±9 | 26±18 | 26±21 | 17±15 | 29±21 | 20±14 | 47±28 | 5±5 |
| Range | 62 93 | 6–64 | 4 58 | 3 50 | 1 57 | 2 37 | 3–94 | 1 18 |

(table IV) contained 21% of sheep-erythrocyte rosetting cells within the nodules and 90% of cells were fluorescent

The receptor pattern in 11 cases of diffuse lymphoma (table V) is different from the nodular variety Cases 1–5 show a predominance of fluorescent cells indicating a B cell origin The presence of low numbers of IgG and EAC receptor bearing cells in association with a high percentage of surface Ig positive cells (case 2) is again noted Case 7 was the only one showing a *predominance of T cells Case 9 is important because unequivocal histology* revealed a uniform and homogenous picture in which equal numbers of T and B cells were recorded Receptor silence was present in case 10 Case 11 represents a repeat biopsy from case 1 of the nodular lymphoma series where the histology had changed from the nodular to the diffuse form Low numbers of cells expressing IgG and EAC receptors were found in both biopsies

## *Discussion*

The receptor pattern of our 8 cases of nodular lymphoma fall largely within normal limits similar findings were reported by JAFFE *et al* [4] These

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# Acute Disseminated Lymphosarcoma with B Cell Markers in a Child

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*Key Words.* B cell markers · Immunology of malignant lymphoma · Lymphosarcoma · Meningeal leukemia

*Abstract.* A case of a 4-year-old child with disseminated lymphosarcoma with meningeal involvement is described. Immunological investigations of the blast cells in the cerebrospinal fluid were carried out and in most of them Ig determinants and no response to PHA stimulation were found. So the B-dependent nature of these cells was ascertained. This observation is a contribution to the diagnosis of lymphoid malignancies through the evaluation of the immunological nature of the blast cells. The data of the literature are still scarce and inconclusive.

In this study, the B dependent nature of the cells of a disseminated lymphosarcoma occurring in a 4-year old child is demonstrated by the lack of response to PHA and by the presence of Ig receptors in nearly all the cerebrospinal fluid (CSF) cells available during a meningeal invasion.

## Case Report

The patient is a 4-year-old boy first seen in our Department on November 27, 1972. A short time before, he had had a small, hard swelling in the left supraorbital region. A biopsy was made and sent to us.

On admission, the physical examination showed only pre-auricular and submandibular lymphadenopathy. Red blood cell count: 4.2 million/μl, hematocrit: 38%, hemoglobin: 11.8 g%, platelet count: 270 000/μl, white blood cell count: 5 000/μl with 49% neutrophils, 47% small lymphocytes, and 4% monocytes. ESR, serum Ig, blood urea nitrogen, serum electrolytes, urinary catecholamines, a bone marrow aspirate, skeleton X-rays were all within normal limits.

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## *Case Report*

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On admission, the physical examination showed only pre-auricular and submandibular lymphadenopathy Red blood cell count 4.2 million/µl hematocrit 38%, hemoglobin 11.8 g% platelet count 270 000 µl white blood cell count 5,000 µl with 49% neutrophils 47% small lymphocytes, and 4% monocytes ESR, serum Ig, blood urea nitrogen, serum electrolytes, urinary catecholamines, a bone marrow aspirate, skeleton X-rays were all within normal limits

In the above-mentioned biopsy, striated muscular tissue and adipose tissue were heavily infiltrated by monomorphous cells showing round nuclei with a dense chromatin pattern and a scarcely visible and coarse cytoplasm, sometimes appearing as a halo. An infiltration of immature lymphoid cells was diagnosed.

A diagnosis of regional lymphosarcoma was then made and antiblastic therapy started. Vincristine (1.5 mg/m²) was administered on a weekly schedule and cyclophosphamide (100 mg/m²) daily, while a radiotherapy course of 3,000 R was completed on the site of the biopsy. The boy was treated as an outpatient until June 1973, when he was admitted again because of increasing pallor. Red blood cell count was then 3 million/μl, hematocrit, 25%, hemoglobin, 7.8 g%. A bone marrow aspirate showed a nearly complete substitution of the normal cell population by atypical lymphoid cells. A diagnosis of disseminated lymphosarcoma was made and a more aggressive antiblastic polychemotherapy started (thioguanine, rubidomycine, arabinoside cytosine, methotrexate, prednisone, cyclophosphamide, vincristine: TRAMPCO, every 2 weeks). A complete remission was promptly obtained: on September 18, a bone marrow aspirate revealed a normal cell population. The cyclic polychemotherapy was continued and the child was well until March 1974, when he suddenly presented with headache, vomiting and strabismus. The physical examination showed mydriasis, reduced light reflex of the pupils, right 6th and left 7th nerve palsy, impaired swallowing reflex, bilateral Babinski's sign, cranial tympanism and other symptoms of meningeal irritation. A funduscopic examination, EEG and skull X rays confirmed the clinical diagnosis of increased intracranial pressure. A lumbar puncture showed an increased pressure of the CSF which contained a small number of blasts (36/μl). A therapeutic regimen including cranial irradiation ($^{60}$Co, 2,400 R in a 4-week period) and intrathecal methotrexate (7.5 mg/m² weekly) was established. Symptoms of meningeal involvement rapidly improved and now, after 8 months, the child is in marrow remission and his spinal fluid appears normal. He is still on intrathecal methotrexate and TRAMPCO.

*Immunological investigations.* CSF cells and peripheral blood lymphocytes, separated on a Ficoll Hypaque gradient, were tested against an anti human globulin (IgG+IgA+IgM) antiserum labeled with fluorescein isothiocyanate (Behringwerke). The response rate to *in vitro* stimulation with PHA was also evaluated. Most of the CSF lymphoid cells showed Ig determinants (80%) and a lack of response to PHA stimulation. In peripheral blood an impaired phytoresponsiveness and an increased rate of fluorescent cells have been observed (65%) (table I), the latter results being in sharp contrast with the low values of serum Ig (IgG: 400 mg%, IgA: 75 mg%, IgM: 30 mg%). As CSF cells were so scarce, immunofluorescence tests could not be performed either with monospecific or with anti κ- and anti λ-chain sera.

## *Discussion*

The results clearly demonstrate the B-dependent nature of the blast cells. The study of the surface markers of lymphoid cells is of greatest usefulness not only for understanding normal immunity, but also in the analysis of neoplasia affecting the lymphopoietic system [12]. As for pediatric patients

*Table I*

| Source of the cells | B lymphocytes, % | Control cpm | PHA M 0 01 ml ml cpm | PHA / control |
|---|---|---|---|---|
| CSF | 80 | 2,610 ± 450 | 4 080 ± 931 | 1.56 |
| Peripheral blood | 65 | 3 060 ± 339 | 49,575 ± 9,026 | 16.20 |

Control = unstimulated lymphocytes, PHA M phytohemagglutinin M-(Difco) stimulated lymphocytes, cpm counts per minute (results are expressed in cpm as the mean value of replicate cultures after incubation with ³H thymidine), $\frac{\text{PHA}}{\text{control}}$ ratio of stimulated to unstimulated lymphocytes

only, pathological cells of subjects with acute lymphocytic leukemia (ALL) have been found to possess in general impaired markers either for B or T cells [2, 4, 8, 14, 16, 18] Recently, however, cases of ALL have been reported, in which blast cells were able to bind sheep erythrocytes or to react with antisera to thymocytes, therefore suggesting involvment of T cells [3, 10–12, 14] Most of the above mentioned investigations have been done on CSF cells because only in such conditions no contamination from normal cells occurs

The immunological characteristics of blasts in childhood malignant lymphomas are scarcely known, but, with a single exception, a T derivation seems to be demonstrated in the cases described untill now [5, 9, 11] The only exception was a 14-year-old patient, described by Gail-Peczalska *et al* [7] who developed leukemia during an abdominal lymphoma The presence of Ig molecules on the surface of his blast cells clearly suggested an involvment of the B dependent system Kaplan *et al* [9] reported 4 cases whose cells – a suspension of tumor or CSF cells collected during meningeal involvement – had a very high rate of E rosette forming cells and an almost complete absence of receptors for membrane Ig and complement (EAC rosette) A T derivation has been looked upon by Castleberry *et al* [5] in 2 cases of lymphosarcoma who first developed acute leukemia and subsequently CNS involvement More than 99% of the cells reacted with antisera prepared with lymphoid cells of agammaglobulinemic patients or with fetal thymocytes This is in contrast with the observations of adult lymphosarcomas which are frequently of B-dependent nature [1, 6, 17]

Our data are complementary to the previous reports of the pediatric literature It is evident that identical possibilities of B or T involvement exist if malignant lymphoma has to be considered as a cancer of the lymphoid system [9]

The practical and theoretical interest of the study of lymphocyte surface markers has been recently emphasized by KAPLAN *et al* [9] who point out its usefulness in the differential diagnosis between ALL and malignant lymphomas which is not always easy on clinical and hematological ground The true value of this method of investigation has been, however, invalidated by the recent observation of cases of T cell ALL and by the possible existence of B cell ALL's [3, 4, 10–13, 15] Moreover, some considerations come out from the evaluation of the rate of fluorescent cells in peripheral blood of our patient during a complete marrow remission The demonstration of a higher than usual quantity of B lymphocytes stands for a possible monoclonal origin of at least some morphologically normal cells. The study of surface markers thus seems to us a new substantial parameter for the evaluation of the situation of rest or activity of the disease

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# The Action of Seed and Other Reagents on HEMPAS Erythrocytes

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*Key Words* Blood groups Congenital dyserythropoietic anaemia HEMPAS Hereditary anaemias Lectins Seed agglutinins

*Abstract* Erythrocytes of a patient with hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS) were tested with many seed extracts and various other reagents Serological evidence of membrane abnormality was confirmed Various anti H reagents reacted relatively poorly with HEMPAS cells HEMPAS cells have both enhanced i and depressed H antigens A brief note on a 'new' anti H lectin (*Cytisus glabrescens*) is provided.

Hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS), or congenital dyserythropoietic anaemia, type II, is an inherited (autosomal recessive) anaemia in which there is a structural abnormality of the red cell membrane [6, 7, 14] which may be responsible for defective cell division, failure of the multinucleated erythroblasts to expel their nuclei and for characteristic serological reactions HEMPAS red cells are agglutinated at 20 °C by about one in three normal (acidified) sera, which contain a naturally occurring IgM alloantibody, anti-HEMPAS, which can be specifically absorbed by HEMPAS cells In the presence of complement, this antibody lyses HEMPAS cells The antibody is not present in the sera of HEMPAS patients The amount of lysis depends on the strength of the antibody, the temperature of incubation and the serum-to-cell ratio [6] Because HEMPAS red cells are agglutinated by many human sera, irrespective of blood group, they may be classified as polyagglutinable The i-antigen strength of HEMPAS is greatly enhanced and may exceed the i antigen strength of newborn infants The strength of the I antigen may be either within normal limits or increased, depending on the source of anti-I, and HEMPAS cells are readily prone to lysis by anti-I and anti-i in the presence of complement

Because seed agglutinins have been usefully applied to the study of various red cell membrane anomalies and to the elucidation of various forms of erythrocyte polyagglutination [1, 4], we have studied the action of seed and other reagents on HEMPAS erythrocytes

## *Materials and Methods*

Seed extracts were prepared and tested by standard methods [2–4] HEMPAS erythrocytes were obtained through the kindness of Mrs. MARIE CROOKSTON of Toronto (4 samples) and Dr A. GERBAL of Paris (3 samples) All tests were carried out on the cells of M F (group O) the patient described by CROOKSTON *et al* [7] The other HEMPAS cells were used to confirm apparently significant observations made on the cells of M F Appropriate normal control cells were tested throughout. Preliminary tests for avidity (speed of reaction by the tile method) were done first, and titre scores by the tube method were determined whenever there were significant differences in avidity In our experience, avidity of agglutination on a tile often gives a sharper indication of seed agglutinin specificity than titres or titre scores obtained in tube tests

## *Results*

Tests on M F were carried out in 1973 and the confirmatory tests on other HEMPAS cells in 1974 and 1975 The results are recorded in tables I–V Since one of the blood group-specific seed agglutinins usefully applied in this study has not been previously reported, we have included a brief description of it at the end of this paper The important observations on HEMPAS erythrocytes were as follows (1) no specific seed anti-HEMPAS reagent was found, (2) the greatly enhanced reactions of HEMPAS cells with anti i sera were confirmed, (3) the I antigen strength of our patient was within normal limits, (4) the H antigen as measured by several anti H reagents was clearly shown to be depressed This was particularly noticeable with the anti H agglutinins from *Cytisus sessilifolius* seeds and from the eel (*Anguilla anguilla*) which react much less avidly and have lower titer scores with HEMPAS than with normal cells, (5) some 'non specific' seed reagents, e g *Maclura aurantiaca* and *Wistaria sinensis* reacted more strongly and had higher titre scores with HEMPAS than with control cells and (6) the agglutinin from the albumin gland of the snail, *Helix pomatia* weakly agglutinated group O HEMPAS cells, but not control cells

*Table I* Reagents which acted with about equal avidity with HEMPAS and control cells

Aggregators polybrene, Alcian blue, ruthenium red, lanthanum nitrate, protamine, polylysine, DEAE-dextran, methylcellulose

Lectins/protectins *Amaranthus caudatus, Amaranthus hypochondriacus, Cytisus glabrescens*[1], *Datura fastuosa, Datura metelloides, Erythrina indica, Helix (Cepea) hortensis, Lens esculenta, Sophora japonica, Solanum tuberosum, Tamarix gallica, Viscum album*

Human sera anti I

[1] Had lower titre score however (table V)

*Table II* Reagents with greater avidity for HEMPAS than with control cells

'Non specific' lectins *Clerodendron trichotomum, Erythrina christa-galli, Lathyrus tingitanus, Maclura aurantiaca, Orobus vernus, Phaseolus caracalla, Phaseolus multiflorus Pisum sativum, Quercus robur, Ricinis communis, Robinia viscosa, Sambucus nigra, Triticum vulgare, Wistaria sinensis*

Human sera anti i

Specific lectins/protectins *Bauhinia variegata* (anti N) (M F is group NN), *Cerastium tomentosum* (anti H)[1], *Evonymus europaeus* (anti B+H)[1], *Helix pomatia*[2] (anti A cross reacts with neuraminidase-treated B or O cells), *Moluccella laevis* (anti A+N) (M F is group NN), *Petteria ramentacea* (incomplete anti H), *Ulex europaeus* (anti H inhibited by L fucose)[1]

[1] Had lower titre scores however (table V)
[2] Agglutinated M F cells weakly, no reaction with control O cells

*Table III* Reagents which reacted less avidly with HEMPAS than with control cells (anti H)

*Anguilla anguilla*[1], *Cytisus sessilifolius*[1], *Laburnum alpinum, Lotus tetragonolobus*

[1] Appreciably poorer avidity

## Discussion

The remarkable enhancement of the i antigen in the presence of a normal amount of I, which was confirmed in our studies, contradicts the belief once held that there is a reciprocal relationship between I and i This contradiction is not confined to HEMPAS, it is also seen in other anaemias associated with bone marrow stress, e g thalassaemia major [10]

*Table IV* Reagents negative with both HEMPAS and controls, i e no HEMPAS-specific reagent found

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Lectins *Acacia dealbata, Adonis aestivalis, Agastache mexicana, Akocanthera spectabilis, Anchusa capensis, Angelica archangelica, Anthyllis montana rubra, Asclepias eurassavica, Asparagus meyersii, Aucuba japonica, Basella rubra, Beta vulgaris, Bignonia tweediana, Caccalia cochinea, Calpurnia aurea* (anti-A + B), *Cardiospermum halicacabum, Carica papaya, Catalpa bignonioides, Catananche coerulea, Ceanothus americana, Cladrastis tinctoria, Clarkia elegans, Coleus candida, Cordyline indivisa, Corokia cotoneaster, Coronilla emerus, Cotyledon simplicifolia, Cuphea lanceolata, Cyphomandra betacea, Cytisus maderensis, Cytisus nigricans, Cytisus supinus, Datura suaveolens, Dicentra spectabilis, Didiscus coerulea, Digitalis mertonensis, Dolichos biflorus* (anti-A1), *Echium plantaginium, Echium rubrum, Elscholtzia stauntonii, Eruca sativa, Euphorbia lathyrus, Fatsia japonica, Gaura lindheimerii, Gingko biloba, Gleditschia triacanthus, Glycine soja, Griselinia littoralis, Gypsophila pacifica, Helianthus annuum, Holboiella latifolia, Holmskildia sanguinia, Horminum pyrenaicum, Hovenia dulcis, Humulus japonicus variegatus, Hyssopus aristatus, Iberis coronaria hyacinthifolia, Iberis jucunda, Iberis sempervirens, Iberis umbellata, Ionopsidium acaule, Isatis tinctoria, Kaulfussia amelloides, Lagenaria leucantha, Lavendula spica, Layia elegans, Leonotis leonuris, Leontopodium alpinum, Leschenaultia biloba, Lespedeza sieboldii, Leucospermum bolusii, Liatris pycnostachya, Limnanthus douglasii, Linum alpinum, Linum utilitissimum, Lonicera caprifolium, Lotus corniculata, Luffa, Lupinus speciosa, Lychnis coeli-rosa, Martynia louisiana, Medicago echinus, Melia azederach, Monarda didyma, Nepeta macrantha, Nicandra physaloides, Ocimum minimum, Omphalodes linifolia, Papaver somniferens, Paronychia capitata, Perilla frutescens, Phacelia campanularia, Philodendron bipinnatifidum, Phlomis samia* (anti-A + B), *Phlomis viscosa* (anti A + B), *Phytolacca americana, Picnostachys urticifolia, Pueraria lobata, Quamoclit lobata, Rumex scutatus,* [illegible] (anti-Tn), *Salvia splendens, Sanguinia canadensis, Saponaria calabrica, Sarcococca con-*[illegible] *Thymus serpyllum, Torenia fournieri grandiflora, Verbascum bombyciferum, Viscaria candida, Zea mays*

Human sera 'incomplete' Rhesus antibodies e g. anti-D with HEMPAS D + ve cells in saline

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*Table V* Titre scores with various reagents

| | HEMPAS | Control |
|---|---|---|
| Anti H reagents | | |
| *Anguilla anguilla* (anti H) | 16 | 38 |
| *Cerastium tomentosum* (weak extract) | 5 | 10 |
| *Cytisus glabrescens* | 23 | 44 |
| *Cytisus sessilifolius* | 25 | 47 |
| *Evonymus europaeus* (anti B+H) | 44 | 47 |
| Human anti H (Bombay plasma) | 13 | 21 |
| *Laburnum alpinum* | not tested, extract too weak | |
| *Lotus tetragonolobus* (weak extract) | 7 | 13 |
| *Ulex europaeus* | 32 | 51 |
| Miscellaneous | | |
| *Maclura aurantiaca* (syn *pomifera*) | 92 | 77 |
| *Wistaria sinensis* | 46 | 16 |

and malignant disorders of lymphoreticular tissues [11] An explanation must await the full chemical characterization of I and i and elucidation of their biosynthesis

A relationship between I, i and H is already known [2] Our observations show that in HEMPAS cells there is a reciprocal relationship between H and i ROCHANT *et al* [12], independently tested the red cells of a HEMPAS patient with an extract of *Ulex europaeus* seeds, with negative results, but observed that anti-H was not present in the patient's serum We do not know whether the H antigen of their patient's red cells was very much more depressed than those of our patients, or whether their *Ulex* extract was very weak We confirmed the weak agglutination of HEMPAS cells by the *Helix pomatia* protectin reported by BIZOT and MONIS [5] The *Helix hortensis* protectin, however, reacted about equally with HEMPAS and control cells

Early in our investigation, we confirmed that HEMPAS cells were different from other known forms of polyagglutinable erythrocytes T, Tk, Tn, which are acquired characters, and Cad (Cad 1) which is an inherited form of polyagglutination The non-identity of HEMPAS with any of these forms of polyagglutination was demonstrated by cross-absorption studies and by the reactions of various seed reagents For example, HEMPAS cells are not agglutinated by the lectin of *Arachis hypogaea* (anti-T)

or *Salvia sclarea* (anti-Tn), *Salvia horminum* (anti-Tn + anti-Cad) or *Dolichos biflorus* (anti-$A_1$, anti-Tn and anti Cad) [1, 4] CROOKSTON and CROOKSTON [8] have shown that HEMPAS is different to T, BIZOT and MONIS [5] that it is different to Cad, and ROCHANT *et al* [12] that it is not T, Tn or Cad

The En(a–) character, in which there is a red cell membrane defect, was also distinguished from HEMPAS En(a–) cells are not polyagglutinable They are agglutinated in saline by 'incomplete' Rhesus antibodies and by the lectin of *Sophora japonica* [9] We found that HEMPAS cells (M F) were not agglutinated either by 'incomplete' Rhesus antibodies in saline or by the *Sophora japonica* lectin which reacts strongly with En(a–) cells or those of En(a+) heterozygotes, this was first observed by SANGER [13] who also observed that these cells in saline do not react with incomplete anti D, unlike En(a–) or En(a+) heterozygous cells, that they reacted normally with rabbit or human anti $En^a$ and reacted more strongly than a normal NN control with *Vicia graminea* and *Bauhinia purpurea* lectins Furthermore, the *Maclura aurantiaca* lectin, which reacts relatively poorly with En(a–) cells [3], reacts more strongly with HEMPAS cells (table V)

*Brief note on a 'new' anti-H lectin* Because of the difficulty sometimes experienced in obtaining seeds of various plants for the preparation of blood grouping reagents, it is always useful to learn of new ones In March 1973, during one of our periodic searches for 'new' lectins, we found an anti H agglutinin in the seeds of *Cytisus glabrescens* Extracts of the seeds reacted on red cells in the following descending order of avidity O, $A_2$, B, $A_1$ $O_h$ cells were not agglutinated Titre scores were as under O = 56, $A_2$ = 52, B = 25, $A_1$ = 10 The avidity and titration results are typical of anti H [2] The agglutinin was inhibited by secretor, but not non secretor, saliva L-fucose did not inhibit, *N*-acetylglucosamine inhibited only slightly, but salicin neutralized the lectin (titre 32) Thus the *Cytisus glabrescens* anti H agglutinin is of the *Cytisus sessilifolius* type, not the *Lotus tetragonolobus* eel serum type of anti H, which is neutralized by L-fucose It is quite a good anti H and would be suitable for routine laboratory use The *Cytisus glabrescens* agglutinin, as did other anti H reagents reacted relatively poorly with HEMPAS cells (table V)

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# Combined Hereditary Deficiency of Factors VII and VIII

## A Distinct Coagulation Disorder Due to the 'Lack' of an Autosomal Gene Controlling Factor VII and VIII Activation?[1]

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*Key Words* Blood coagulation Coagulation disorders Factor VII and VIII deficiency

*Abstract* A patient with a combined hereditary deficiency of factors VII and VIII is presented together with a family study The main bleeding manifestations were easy bruising and bleeding after tooth extractions. No hemarthrosis was ever observed. The main laboratory features consisted in a mild prolongation of prothrombin time and of partial thromboplastin time TG test was abnormal and was corrected by the addition of adsorbed normal plasma Specific assays revealed a moderate defect of factors VII and VIII All other clotting factors were within normal limits. The factor VII antigen in the propositus was normal or nearly normal The factor VIII-associated antigen was also normal Five additional family members presented the same coagulation pattern and were variably symptomatic The hereditary transmission pattern seems to be autosomal dominant. The defect appears to be due to a structural abnormality of a gene controlling factors VII and VIII activation

Hereditary deficiencies of more than one clotting factor are very rare coagulation disorders The only condition which seems to be an indipendent nosological entity is combined deficiency of factors V and VIII About 20 patients with such a peculiar defect have already been satisfactorily documented [9, 12, 17, 24 27] Similar considerations could be formulated for combined deficiencies of the prothrombin complex factors [4, 23] But the matter is still disputed particularly because only 2 cases have been described and because the defect seemed to be correctable almost completely by parenteral administration of vitamin K [4, 23] Therefore, the possibility of an acquired defect secondary to impaired vitamin K adsorption remains

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